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GROWTH OF SMALL NUMBERS OF TUBERCLE BACILLI, H37, IN LONG'S LIQUID SYNTHETIC MEDIUM AND SOME INTERFERING FACTORS

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Very small amounts of substances inhibiting the growth of amounts less than about 10^{-2} mgm. of well-dispersed and suspended tubercle bacilli can be readily and unknowingly introduced into Long's synthetic medium.

This fact is of great importance when investigating probable growth factors for these bacilli. It is of equal importance in *in vitro* studies of the probable inhibiting or lethal effects of substances deliberately added to the culture medium.

If injurious contaminating agents are kept out of Long's liquid synthetic medium, growth of the strain, H37, of human tubercle bacilli will result from amounts at least as small as 10^{-7} to 10^{-8} mgm.

If contamination occurs, growth may be limited to somewhere between 10^{-1} and 10^{-7} mgm. depending upon the amount and kind of contamination.

I

Drea (1940) reported that amounts of the H37 strain "at least as small as 10^{-6} mgm." could be cultured in a slightly modified form of Long's medium. It seemed unnecessary to discuss the self-evident irregularities recorded in a table in that paper for growth from the 10^{-6} to and including 10^{-10} mgm. plantings, since the series was a brief preliminary one, and no conclusions were drawn regarding growth with less than 10^{-6} mgm. However the failure to discuss this point seems to have made the table appear misleading. The present article will clarify the matter.

Continuation of the research following the observations already reported resulted finally in noting that growth of the bacilli was limited to amounts greater than 10^{-6}, 10^{-5}, 10^{-4} or 10^{-3} mgm. with the same technique, in general, as previously used.

The most probable explanation was that contaminating substances inhibiting growth were now present that were previously absent.

The glassware at the beginning of the research was new and after each growth experiment was cleaned and prepared in the usual way of this laboratory for the next plantings of bacilli. It seemed probable, therefore, that injurious invisible films on the glass surfaces had been gradually built up in the tubes used to grind and suspend the bacilli, in the flasks containing the culture medium and in the pipettes.

Elimination of these possible sources of contamination has resulted in demonstrating that growth of the bacilli will result in Long's synthetic medium after estimated 10^{-7} and 10^{-8} mgm. amounts have been planted by taking 0.1 ml.

of 10^{-6} and 10^{-7} mgm. per ml. dilutions respectively. (See Technique, Section IV.)

II

Sources of contamination

Four probable sources of contamination were as follows: (1) persistent adsorption by the glass of soap and other organic films incidental to the cleaning of the glassware in spite of repeated final rinsings with hot tap water; (2) adsorption of the paraffin used to lightly seal the cotton stoppers after planting the bacilli; (3) adsorption of distillates from the bleached non-absorbent cotton used as stoppers for the tubes, flasks and pipettes; and (4) adsorption of metabolic products produced by the tubercle bacilli.

Emphasis is laid upon this fact: that the glassware after the final rinsing with hot water and when dry appeared to be thoroughly clean. There were no visible adsorbed films present.

III

Technique for preparing flasks containing synthetic medium free from contaminating adsorbed films

The glassware must be thoroughly cleaned. When thought advisable, a saturated solution of KNO_3 in concentrated H_2SO_4 was used as a cleaning agent. Bichromate-acid solution was not used because of the possible introduction of chromic compounds (reaction products) into the glassware.

Bleached non-absorbent cotton stoppers when sterilized with the flasks in the hot air oven and autoclave were demonstrated to give off growth-inhibiting distillates. Because non-bleached non-absorbent and bleached absorbent cotton also give off distillates when heated, the use of cotton has been discontinued in this work.

Long's synthetic medium (Long and Seibert, 1926) was used with 2.33 grams of anhydrous Na_2CO_3 per 1000 ml. instead of the amount called for in the original formula. The pH was about 7.1 after autoclaving. Twenty ml. of the medium were placed in each 50 ml. Erlenmeyer flask to be planted.

As substitutes for cotton stoppers, either plain transparent Cellophane, No. 300, well washed with distilled water, was fastened with cleaned rubber bands over the tops of the flasks, or aluminum foil of thickness, 0.00065 inch, previously held over a Bunsen flame, was folded over the tops of the flasks, or flame-cleaned, loosely fitting aluminum cylinders were placed over the tops. As compared with loosely fitting Pyrex glass caps the cellophane and aluminum proved to be equally inert. The same method of capping was used for the parent cultures from which the transplants were secured and for the stock solution from which the culture flasks were filled. The flasks with the culture media were then autoclaved at 120° C. for 20 minutes.

Pyrex glassware, except for the pipettes, was used.

IV

Technique for preparing bacterial suspensions and planting the bacilli

All procedures were planned to avoid contamination by other microorganisms. The air currents in the room were kept to a minimum for several hours before planting the bacilli.

Some of the three-weeks-old surface growth from a vigorously growing culture of the H37 strain on Long's medium was removed and dried on fat-free well-washed absorbent paper. About 6 mgm. were weighed to within 0.1 mgm. in the grinding tube. The grinding tube was a rimless Pyrex tube of 15 x 125 mm. outside dimensions, into the bottom of which was ground, with the aid of medium coarse carborundum powder, one end of a 28 x 1 cm. Pyrex glass rod, thus providing good mortar-pestle, closely fitting, rather rough grinding surfaces. The technique for grinding, suspending and preparing diluted suspensions was in general that of Corper and Cohn (1936) using a 0.5 per cent solution of sodium taurocholate (Eastman, practical) in water for grinding the bacilli. The suspending fluid was 0.8 per cent NaCl in water, Long's medium or Long's medium with the magnesium sulphate, ferric ammonium citrate and glycerol omitted. Each of these solutions was satisfactory.

The grinding was done by hand. The first suspension was of 1.0 mgm./ml. It was thoroughly mixed and allowed to stand for five minutes, when 0.5 ml. was transferred from the middle of the suspension to 4.5 ml. of the fluid in the next decimal dilution tube. Decimal dilutions down to the desired limit were made.

A fresh 1.0 ml. pipette was used for each dilution and before taking up the suspended bacteria its interior was washed once with suspension fluid from a separate flask. Transferrence of possible surface bacterial films was avoided by wiping the end of the charged pipette with oil-and-fat-free linen. The suspension was then slowly discharged into the next dilution tube not allowing the pipette to touch the tube or its contents. Agitation of the new suspension was done by a fresh pipette before withdrawing the desired amount to be transferred to the next tube.

Pasteur pipettes, prepared and calibrated as described by Fildes (1931), were used for planting the bacilli. Three drops (0.1 ml.) of the bacillary suspension were slowly dropped from above the surface into the medium. The pipettes were first washed with suspension fluid to which no bacilli had been added and a fresh one was used for each decimal dilution. The estimated amounts of planted bacilli ranged from 10^{-1} mgm. in decimal steps to the smallest amount to be planted. The bacilli were shielded from direct light rays when suspended and planted.

After planting, aluminum foil of thickness, 0.00065 inches, was flamed over a Bunsen burner and folded over the top and neck of the flask. The aluminum foil cap was not sealed to the glass with paraffin or any other substance. Cleaned filter paper, sealed with paraffin, was used to cap the planted flasks in the early experiments.

The planted flasks were then placed in the incubator at 37.5°C.

V

Results

During the past fifteen months twenty-eight separate plantings were made using sodium taurocholate as the dispersing agent for the bacilli. The plantings were mostly controls for other investigations under way. Four of these plantings were made in 2 parallel rows of flasks, two in 3 parallel rows of flasks and one in 5 parallel rows. The other twenty-one plantings were in single rows.

Plantings were made from decimal dilutions calculated to give 10^{-1}, 10^{-2}, etc. mgm. bacilli.

Of the four 2-parallel-row plantings, one resulted in growth to 10^{-8} mgm. bacilli in each row, two resulted in growth down to 10^{-7} mgm. in one row and 10^{-8} mgm. in the other, and the fourth planting resulted in growth in one row to 10^{-8} and in the other to 10^{-9} mgm.

TABLE 1

NUMBER OF TIMES SMALLEST AMOUNT OF BACILLI GREW	MGM. OF BACILLI PLANTED (ESTIMATED)										
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
1	+	+	+	+	+	−	−	−			
1	+	+	+	+	+	+	−	−			
17	+	+	+	+	+	+	+	−			
18	+	+	+	+*	+	+	+	+	−		
2	+	+	+	+	+	+	+	+	+	−	
1	+	+	+	+	+	+	+	+	+	+	−
40 Total											

* There was no growth in one flask. No other break in continuity noted.
+ = growth. − = no growth.

Of the two 3-parallel-row plantings, one resulted in growth in all 3 rows to 10^{-7} mgm. and the other had growth down to 10^{-7}, 10^{-7} and 10^{-8} mgm.

From the one 5-parallel-row planting, there was growth to 10^{-8} in 3 rows and 10^{-7} mgm. in 2 rows.

There were no discontinuities of growth in the above parallel rows accounted for, growth taking place in all flasks planted with amounts greater than the minimum amounts of bacilli from which growth developed.

In one of the 21 single row plantings there was no growth from the 10^{-4} mgm. planting when there was growth from all the others to and including the 10^{-8} mgm. planting in that row.

Altogether, there were 40 plantings (table 1). These are non-selected, consecutive examples.

Figure 1, based on these figures for minimum amounts from which growth resulted, approximates a normal frequency distribution curve.

From the forty plantings, 97.5 per cent resulted in growth from amounts at least as small as 10^{-6} mgm. of the H37 strain, confirming the similar statement

made in my 1940 report. Ninety-five per cent developed growth from amounts at least as small as 10^{-7} mgm. and it appears safe to state that growth may be expected with regularity from some amount between 10^{-7} and 10^{-8} mgm. The surprising growth occuring in two plantings with 10^{-9} mgm. and in one planting with 10^{-10} mgm. was also reported in the previous paper.

The average times of first definite appearances of growth were as follows: 10^{-1} mgm. in 6 days; 10^{-2} in 9 days; 10^{-3} in 10 days; 10^{-4} in 14 days; 10^{-5} in 17 days; 10^{-6} in 20 days; 10^{-7} in 27 days and 10^{-8} in 28 days. One of the 10^{-9} mgm. growths developed in less than 53 days and the other sometime between

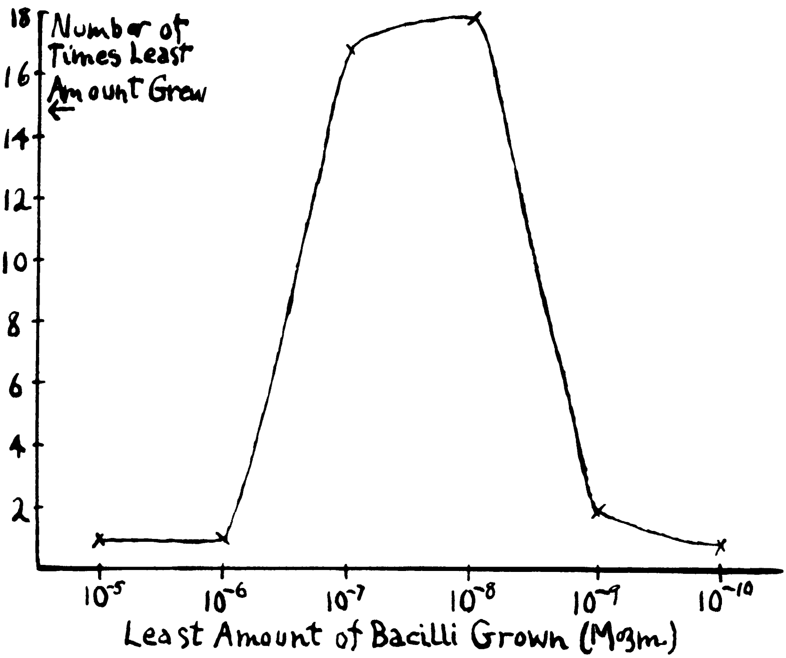

FIG. 1

62 and 76 days. The 10^{-10} mgm. growth developed at some time less than 54 days.

An incubation time of 75 days should be sufficient to establish final results as to presence or absence of growth.

The growth always began at the bottom of the medium Later, growth extended to the surface of the medium from plantings of 10^{-5} mgm. or more of bacilli.

From smaller plantings, there was usually failure of surface growth to develop after the depth growth had been definitely established. Transplanting a small amount of the depth growth when there was failure of surface growth, to fresh synthetic media resulted in renewed growth and later development of surface growth.

The mass of bacilli at the bottom of the medium when there was no surface growth did not exceed about 21.0 mgm. Generally it was less than this amount, when the growth had extended to the surface. When growth was established on the surface there appeared to be no further growth in the depth. The surface growth when most profuse was about 960 mgm. The bacilli at the bottom were acid-fast to the Ziehl-Neelsen stain.

10^{-5} mgm. 10^{-6} mgm. 10^{-7} mgm.

FIG 2. This is a single photograph of 3 flasks to help make more clear, growth from small numbers of tubercle bacilli in Long's liquid synthetic medium.

H37 was the strain used The incubation period was 64 days

The flasks contain growth from 10^{-5}, 10^{-6} and 10^{-7} mgm. bacilli. Growth was also present as a result of plantings with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-8} mgm. bacilli (not photographed)

The 10^{-5} mgm. flask shows a profuse surface growth and part of the bottom growth

The 10^{-6} mgm. flask shows a thin surface growth not quite covering the surface of the liquid and depth growth

The 10^{-7} mgm flask has no surface growth but does have a considerable amount of depth growth

Growth from the heavier plantings is of flocculated form at the bottom of the media. With the smaller plantings there exist at first colonies or clumps which go on to produce a more diffused growth. Because of this, and more especially because of the fluidity of the medium, it now appears more accurate to record the positive findings as "growth" and not as "colonies."

Since sodium taurocholate labeled "practical," had been used as a dispersing agent in making the bacillary suspensions it was decided to use an especially purified sample of the dioctyl ester of sodium sulfosuccinic acid with the trade

name *Aerosol OT* (100 per cent),[1] (Caryl and Ericks, 1939) for the same purpose. Thus a dissimilar, more simple compound, was contrasted with the more complicated and less pure taurocholate. Five separate tests were made. In three tests the limit of growth was 10^{-7} mgm. bacilli and in two tests the limit was 10^{-8} mgm. All flasks with amounts greater than 10^{-7} mgm. and 10^{-8} mgm. respectively developed growth. The taurocholate and the dioctyl ester were equally effective as dispersing agents.

The effect upon growth of the bacilli when these two dispersing substances were added to the synthetic medium in decimal dilutions from 10^{-1} to 10^{-7} per cent was observed. In each flask was planted 10^{-3} mgm. (about one million) bacilli. Two separate tests were made on each. The taurocholate in the first test and the dioctyl ester in the other were used as dispersing agents for the bacilli. With each substance in both tests, there was no growth with concentrations of 10^{-1} and 10^{-2} per cent. These two dispersing agents, when added to the synthetic medium, had the same effect on the growth of 10^{-3} mgm. bacilli.

Since, however, growth of the relatively large amount of 10^{-3} mgm. bacilli was inhibited by some amount between 100 and 10 p.p.m. of both substances, it seemed possible that these dispersing agents exert an inhibiting effect upon small numbers of bacilli when they are used only as dispersing agents and that growth could be demonstrated from less than 10^{-7} to 10^{-8} mgm. (estimated) if dispersing agents with a lesser inhibiting growth power were used.

Long's synthetic medium of pH 8.0 as a dispersing agent for the bacilli was tried three times. Growth resulted down to 10^{-6} mgm. in one experiment, down to 10^{-7} in a second, and 10^{-7}, 10^{-8} and 10^{-9} in a third.

Gelatin as the dispersing agent in one test resulted in growth down to 10^{-8} mgm. bacilli.

An M/15 solution of Na_2HPO_4 as the dispersing agent resulted in growth down to 10^{-7} mgm. bacilli in two tests.

A diverse group of agents for dispersing the bacilli thus resulted in similar numbers of the bacilli producing growth and ruled out any specific property of the taurocholate in growth promotion.

The suspensions of 1.0 mgm./ml. for the experiments recorded above were allowed to stand for five minutes to permit clumps of bacilli to settle before transfers of bacilli were made for the next decimal dilution.

This then should actually result in smaller amounts of bacilli being transferred than those indicated by the recorded figures.

The suspending of the bacilli results only in an approximate approach to the ideal suspension of isolated bacilli and many clumps of two or more bacilli must be present.

The effect of not permitting the clumps to settle was now studied. The same technique as before, with sodium taurocholate as the dispersing agent was used, except that when the first 1.0 mgm./ml. suspension had been made and agitated,

[1] The especially purified sample of Aerosol OT, 100 per cent, was provided through the courtesy of Dr. G. B. Ayers of the American Cyanamid Company.

the required amount of this suspension was immediately transferred and decimal dilutions down to 10^{-9} prepared.

Plantings were now made in six parallel rows of flasks.

Four of the rows produced growth down to 10^{-8} mgm. and two down to 10^{-7} mgm. at the end of 75 days incubation. Apparently it did not make any difference whether or not the first suspension was allowed to settle for five minutes before the first dilution was made.

It appears then to be established that growth of the strain, H37, of human tubercle bacilli will result with regularity from an amount somewhere between 10^{-7} and 10^{-8} mgm. when planted in Long's synthetic medium.

Corper and Cohn (1933) estimated that 1 mgm. in moist culture contained about 10^9 (one billion) bacilli.

VI

To check the effect of unheated blood serum upon the inhibiting effect of cotton the following experiment was done.

Four rows of flasks were used. The first row contained synthetic medium only and the flasks were not in contact with cotton at any time. The second contained synthetic medium only and the flasks had non-absorbent cotton stoppers in the oven, autoclave and incubator. The third had 5 per cent serum added to the autoclaved synthetic medium and the flasks were not in contact with cotton at any time. The fourth had 5 per cent serum added to the autoclaved synthetic medium and the flasks had cotton stoppers in the oven, autoclave and incubator. Each row of flasks was planted with decimal dilutions of the tubercle bacilli with amounts from 10^{-1} to 10^{-6} mgm. After planting, the cotton stoppers were lightly impregnated with paraffin and the flasks without cotton were capped with aluminum foil and were not sealed with paraffin. The result was that the cotton-stoppered flasks with no serum had growth develop only from 10^{-1} and 10^{-2} mgm. bacilli and no growth from the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} mgm. plantings. The other three rows of flasks all developed growth from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} mgm. bacilli.

Since growth occurred also in the synthetic medium without the serum where there had been no exposure to cotton down to the smallest amount planted, it seems reasonable to assume a neutralization of the inhibitory effects due to the cotton as possible in addition to the growth promotion by the serum as postulated by Boissevain (1940).

VII

The technique now in effect makes use of either aluminum cylinders or foil for capping the flasks when autoclaving the culture media and aluminum foil for capping during incubation. Evaporation of water during the prolonged incubation period is not excessive. Any evaporation can be counter-balanced by preliminary addition of 5 per cent water to the medium. Cotton is not used at any time.

DISCUSSION

Long's synthetic medium, because of its simplicity, lends itself well to such studies with tubercle bacilli, especially since it is now established that amounts of the human strain, H37, as small as 10^{-7} to 10^{-8} mgm. will grow in it.

The H37 strain used in these investigations is well conditioned to laboratory growth and is used by many investigators in the field of tuberculosis research.

An index of its virulence was secured during this past year by making subcutaneous injections. In groups of four guinea pigs, 10^{-2}, 10^{-4} and 10^{-6} mgm. of the bacilli resulted in death from advanced tuberculosis with average life durations of 140, 166 and 177 days respectively.

It has generally been believed that less than about 1.0 or 0.1 mgm. tubercle bacilli would not grow in Long's medium if accessory factors were not added, that growth must necessarily take place on the surface and that if a transplant of bacilli sank beneath the surface, growth failed to occur. However, Kahn (1929) stated incidental to his study of the developmental cycle of the tubercle bacillus, "In this way a number of young vigorous forms develop in the shallow depths of the medium and there is less chance of isolating non-viable forms." He had incubated for 4 days, suspensions of H37 prepared by shaking a small amount of growth membrane with glass beads in 4 ml. of Long's medium. He also found it possible to grow a single tubercle bacillus or small groups of from 2 to 6 microorganisms in individual microdroplets of the same medium. It is now established that with fine suspensions, growth always begins at the bottom of Long's liquid medium.

The importance of "depth" growth in liquid media was stressed by Kirchner (1931) who added serum or plasma to a liquid synthetic medium in order to secure it.

Growth of the tubercle bacilli in the depth of the liquid medium is more comparable to that in animal tissues than is surface growth on the culture media. Constant immersion insures a more constant environment than does surface growth where bacilli are superimposed on their fellows and are exposed directly to the atmosphere above. This applies, of course, only before growth has extended to the surface of the fluid synthetic medium.

Other strains of tubercle bacilli, especially more recently isolated ones, may not grow from such small numbers in Long's medium as does the H37 strain. It is with such strains that accessory growth factors may be sought if such are needed by the bacilli. For example, strains that will grow on egg media or in serum from amounts as small as 10^{-6}, 10^{-7} or 10^{-8} mgm. and grow in Long's medium only when much larger amounts are planted undoubtedly require the addition of some other substance or substances to the medium.

New strains to be established from infected tissues or sputa require complex media such as egg or serum. Investigations as to the necessary additional factor or factors that must be added to Long's medium to establish growth there also, may be very profitable.

Mueller (1937) demonstrated the importance of small amounts of pimelic acid

and nicotinic acid and Mueller and Cohen (1937) of B-alanine as growth-promoting factors for the diphtheria bacillus. Wadsworth and Wheeler (1934) reported that they obtained growth from thirteen out of twenty recently isolated virulent strains of the diphtheria bacillus in a synthetic medium which did not contain β-alanine and nicotinic acid.

This same condition may prevail for tubercle bacilli, some strains requiring added accessory growth factors and other strains not.

In order, however, to secure growth from small numbers of tubercle bacilli in Long's medium it is necessary to insure the absence of adsorbed, organic growth inhibiting films from the glassware. Especially inhibiting were the distillates from the bleached non-absorbent cotton stoppers in the flasks. H. Braun (1939) described technique for insuring clean glassware and stated that the cleaned dry flasks are stoppered "mit fettfreier Watte." Fat-free cotton is probably satisfactory as a stopper, but instead of cotton, aluminum cappings for the flasks were used in this work when the harmful effects of cotton were to be avoided. It was demonstrated, however, that blood serum added to the synthetic medium permitted growth of the bacilli when the flasks were stoppered with the cotton. In estimating possible growth promotion, it is necessary to consider the probable neutralization by the added substance of inhibiting substances accidentally present in the culture media. Added blood serum can combine with a poison such as copper to neutralize the growth-inhibiting effect of the latter when the inoculum consists of small numbers of various pathogens (O'Meara and Macsween, 1936, 1937). It was also stated by Gordon and McLeod (1926) that serum protects bacteria against concentrations of amino-acids which would otherwise inhibit their growth. Similarly, the growth promotion of small numbers of tubercle bacilli by serum added to Long's medium in the flasks with the cotton stoppers may be at least partly due to neutralization of inhibitory substances.

There is one other kind of investigation that should be of value using this technique of cultivating tubercle bacilli. The bacteriostatic or lethal effects of known substances added to the liquid synthetic medium can be studied. For example, sodium oleate inhibited the growth of tubercle bacilli when present in the amount of 1 p.p.m. according to a personal communication by Dr. C. H. Boissevain. This also gives an idea of the extreme importance of small amounts of inhibitory substances that may readily be acquired by the glassware incidental to incomplete cleaning as well as to the distillates from the cotton stoppers.

A speculation is now perhaps permissible. What effect, if any, has relatively unclean glassware upon the virulence of tubercle bacilli, when small numbers of them are injected in animals?

Because practically all bacterial diseases result from infection with relatively small numbers of organisms, it would appear that *in vitro* studies of relatively small numbers of bacilli are of importance, especially when the amount of growth factors carried over from the parent culture, either within the cells or from the parent culture medium is much diminished.

Amounts of tubercle bacilli, strain H37, as small as 10^{-7} and 10^{-8} mgm. grow in Long's medium and it appears reasonable to expect growth in exceptional

cases from amounts as small as 10^{-9} and 10^{-10} mgm. dilutions as recorded both in this paper and the previous one by Drea (1940). This is because of the inherent errors due to sampling when small numbers of organisms are involved. See Berg (1941).

Corper and Cohn (1933) consistently obtained growth on good non-synthetic media from diluted suspensions of recently isolated virulent avian tubercle bacilli, after plantings containing calculated amounts of 10^{-9} mgm. Since this was before they introduced sodium taurocholate as a dispersing agent, human cultures could not be so finely dispersed, though they did state that occasionally a final dilution suspension was obtained containing 10^{-9} mgm. per ml. The taurocholate and other wetting agents make possible finer dispersions of the human cultures than those produced when they are not used. It appears reasonable, therefore, to suppose that 10^{-9} mgm. per ml. suspensions of the human strains can now be prepared more often.

Let it be assumed that there are 10^9 bacilli in 1 mgm. of the culture and that we have actually prepared a suspension of 10^{-9} mgm. per ml. or what amounts to the same thing, 100 ml. of suspension fluid containing 100 bacilli (1 bacillus per ml.). There is, of course, a random scattering of the bacilli in the suspension fluid resulting in a non-uniform distribution of the organisms. In such discontinuous distributions Poisson's series of probabilities is of the first importance. The probability chance of a 1 ml. sample of this suspension containing bacilli is as follows: 0 bacilli, 37 per cent; 1 bacillus, 37 per cent; 2 bacilli, 18 per cent; 3 bacilli, 6 per cent; and 4 bacilli, 2 per cent. These figures are based on table 1 of Berg (1941).

If now to the above 10^{-9} mgm. per ml. suspension are added 900 ml. of fluid, the 1000 ml. of the resulting 10^{-10} mgm. per ml. suspension will have on an *average* 0.1 bacillus per ml. The same kind of statistical treatment shows that there is a probability chance of 90 per cent that a 1 ml. sample of this suspension will contain no bacilli; a 9 per cent chance that a similar sample will contain 1 bacillus; and a 0.5 per cent chance that it will contain 2 bacilli.

Thus, even if it is further assumed that each bacillus is viable and capable of producing growth, it can be readily understood there must be irregularities of growth when very small numbers of bacilli are planted. There will be failure of growth after some plantings with 1 ml. samples from the 10^{-9} mgm. per ml. suspension and occasionally there will be growth from similar amounts of the 10^{-10} mgm. per ml. suspension.

Since the last paper was published, it appears to be more accurate to record the growth of the bacilli as "growth" and not as numbers of "colonies" even for the smaller amounts of bacilli planted. Because of the fluidity of the medium the organisms may have their positions changed, either to unite in larger clumps or to disperse into smaller ones.

It was also found preferable not to use the slightly modified form of Long's medium previously employed, which was however a synthetic medium. In the investigation now reported, Long's medium was used with only sufficient Na_2CO_3 to give the desired pH value. The reasons for the change from the modified me-

dium previously used are (1) the better buffer action of Long's medium and (2) its much more general use by other investigators.

No investigations were made of substances that may be contributed by the glass itself.

From this work it appears that one well-known strain of tubercle bacilli will grow in Long's synthetic medium from amounts at least as small as 10^{-7} mgm. whereas previously it was thought that amounts greater than about 10^{-2} mgm. were necessary before growth resulted. The irregularities of growth reported in the first paper for amounts of bacilli from 10^{-6} to 10^{-10} mgm. but from which no conclusions were drawn, are now accounted for by the presence of previously unrecognized growth-inhibiting substances adsorbed by the glassware as well as by the errors necessarily associated with random samples where small numbers of bacilli are planted.

SUMMARY

1. Growth of the H37 strain of human tubercle bacilli in Long's liquid synthetic medium can develop when amounts at least as small as 10^{-7} to 10^{-8} mgm. are planted.

2. Very small amounts of contaminating organic substances, hitherto unsuspected, are readily adsorbed by the glassware, and may act as growth inhibitors. The smallest amounts of these bacilli that will grow when such inhibitors are present varies from 10^{-2} to 10^{-6} mgm.

3. A soap, sodium oleate, will inhibit the growth of tubercle bacilli when present in something greater than 0.1 p.p.m. and less than 1.0 p.p.m. Soaps may be a part of the contaminating adsorbed films.

4. Especially inhibiting to growth are the distillates from bleached, nonabsorbent cotton stoppers in the glassware.

5. The elimination of inhibitory substances from a liquid synthetic medium will provide a valuable means of investigating: (1) accessory growth factors and (2) bacteriostatic and bactericidal properties of deliberately added agents in *in vitro* studies of tubercle bacilli.

6. The elimination of inhibitory substances from culture media is of importance in the cultural studies of other microorganisms and it is probable that organic films including the distillates from cotton stoppers adsorbed on the glassware are inhibitory to the growth of small numbers of these organisms especially in synthetic media which otherwise permit growth. This may also be of importance in virulence studies where small numbers of microorganisms are used.

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BIBLIOGRAPHY

Berg, W. N. 1941 The law of small numbers as applied to virulence measurement. Am. Rev. Tuberc., **43**, 685–691

Boissevain, C. H. 1940 Growth promotion of the tubercle bacillus by serum albumin. Proc. Soc. Exptl. Biol. Med., **44**, 110–112.

BRAUN, H. 1939 Handbuch der Biologischen Arbeitsmethoden, Abt. XII, Teil 2, I, 1-78. Urban & Schwarzenberg, Berlin-Wien.

CARYL, C. R., AND ERICKS, W. P. 1939 Esters of sodium sulfosuccinic acid. Ind. Eng. Chem., **31**, 44-47.

CORPER, H. J., AND COHN, M. L. 1933 The nutrient quality of eggs for growing tubercle bacilli. Am. J. Hyg., **18**, 1-24.

CORPER, H. J., AND COHN, M. L. 1936 A mechanical device for preparing fine suspensions of tubercle bacilli and other microorganisms. J. Lab. Clin. Med., **21**, 428-431.

DREA, W. F. 1940 The growth of human tubercle bacilli, H37, in synthetic medium with and without agar. J. Bact., **39**, 197-205.

FILDES, P. 1931 A system of bacteriology, **9**, 174-176. His Majesty's Stationery Office, London.

GORDON, J., AND MCLEOD, J. W. 1926 Inhibition of bacterial growth by some amino-acids and its bearing on the use of tryptic digests as culture media. J. Path. Bact., **29**, 13-25.

KAHN, M. C. 1929 A developmental cycle of the tubercle bacillus as revealed by single-cell studies. Am. Rev. Tuberc., **20**, 150-199.

KIRCHNER, O. 1931 Zur Veranderlichkeit des Tuberculoseerregers in morphologischer Hinsicht. Beitr. Klin. Tuberk., **77**, 72-77.

LONG, E. R., AND SEIBERT, F. B. 1926 The chemical composition of the active principle of tuberculin. I. A non-protein medium suitable for the production of tuberculin in large quantity. Am. Rev. Tuberc., **13**, 393-397.

MUELLER, J. H. 1937 Pimelic acid as a growth stimulant of *C. diphtheriae*. J. Bact., **34**, 163-178.

MUELLER, J. H. 1937 Nicotinic acid as a growth accessory substance for the diphtheria bacillus. J. Bact., **34**, 429-441.

MUELLER, J. H., AND COHEN, S. 1937 Beta-alanine as a growth accessory for the diphtheria bacillus. J. Bact., **34**, 381-386.

O'MEARA, R. A. Q., AND MACSWEEN, J. C. 1936 The failure of staphylococcus to grow from small inocula in routine laboratory media. J. Path. Bact., **43**, 373-384.

O'MEARA, R. A. Q., AND MACSWEEN, J. C. 1937 The influence of copper in peptones on the growth of certain pathogens in peptone broth. J. Path. Bact., **44**, 225-234.

WADSWORTH, A., AND WHEELER, M. W. 1934 The attenuation and toxin production of the diphtheria bacillus. J. Infectious Diseases, **55**, 123-137.

STUDIES OF THE COMMON AEROBIC SPORE-FORMING BACILLI*

II. Fermentation Reactions in Agar Butt-Slants

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Despite the well-recognized need for simple means of identifying the common varieties of aerobic spore-bearing bacilli little attention has been given to the fermentative reactions of these organisms. Most descriptions mention tests with glucose, lactose, sucrose, and maltose only and the reactions have generally been regarded as of no differential value. Liquid culture media have apparently been used by all writers, (except de Soriano, 1935), and the results have usually been reported merely as "plus," "minus," or "variable." (Bergey, 1939; Ford, 1927; Topley and Wilson, 1936; Lamanna, 1940.)

Our first tests made in Durham fermentation tubes with 35 strains of aerobic spore-bearing bacilli, representing ten of the common varieties, revealed that the fermentation of mannitol but not lactose by a member of this group serves to identify the organism as *Bacillus megatherium, provided it is also known to store fat.* At the same time these tests showed the slowness and uncertainty with which slight or moderate acid production is detectable in liquid sugar media, where so many strains of these bacilli grow in a surface pellicle only, and it seemed likely that other reactions of differential value might appear with use of a more sensitive test procedure. We accordingly repeated the tests, using, in addition to broth, soft agar stabs and also agar slants with deep butts. The latter were recommended many years ago by Conn and Hucker (1920) for the detection of acid production by soil organisms. These authors noted that agar butt-slants serve as delicate indicators of acid formation and, moreover, may reveal a tendency on the part of certain cultures to develop a characteristic zone of acid reaction at a particular place in the medium.

COMPARATIVE FERMENTATION TESTS IN BROTH, SOFT AGAR STABS, AND AGAR BUTT-SLANTS

In order to test the value of agar butt-slants in direct comparison with broth and stab cultures a series of parallel tests was run in these three varieties of media.

Technique

The basis of the culture medium employed was a sugar-free infusion broth. Some of this was converted into semi-solid agar for the stab cultures by addition

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of 0.5 per cent of agar and some into ordinary solid agar for the butt-slants by the addition of 1.5 per cent of agar. Twenty per cent stock solutions of the chemically-pure sugars in distilled water, sterilized by autoclaving at 15 pounds' pressure for 15 minutes, were added aseptically to the basic media, just before tubing, in the proportion of 1 per cent. At the same time brom-cresol-purple indicator (1.6 per cent alcoholic solution) was added in the amount of 1.0 ml. per liter. All of the media were adjusted to an initial pH of 7.0.

Tests were made with one carbohydrate at a time by inoculating each of a group of 17 test organisms into the same basic medium in each of the three forms —broth, stab, and butt-slant. The cultures used for these direct comparative studies included 13 different strains, representing the following 10 common varieties of aerobic spore-bearers: *Bacillus megatherium*, *B. mycoides*, *B. cereus*, *B. subtilis* Michigan, *B. circulans*, *B. brevis*, *B. subtilis* Ford[2], *B. subtilis* Marburg, *B. vulgatus*, *B. mesentericus*. In addition, *Eberthella typhosa*, *Escherichia coli*, *Proteus vulgatus*, and *Alkaligenes fecalis* were included as control organisms.

All cultures were incubated at 37°C for 48 hours and were then placed at room temperature for one week's further observation. The changes in the indicator in each individual tube were closely followed, not only by written descriptions, but by preparing at least once daily full-sized reproductions of each tube in which the exact shades of the indicator color were duplicated with crayon. In this manner tests were carried out in triplicate with glucose, lactose, sucrose, maltose, and mannitol media. In the case of organisms showing minimal reactions the cultures were repeated several more times.

Results

Out of a group of 65 sets of parallel cultures of the various spore-bearing bacilli positive fermentation reactions were recorded in one or more of the three test media in 46 instances. Among these there were 20 in which the fermentative changes in the agar butt-slants were definitely positive after incubation for 24 hours or less while a positive reaction in the broth tubes was not observable before the second to the fifth day of incubation; and there were 13 instances in which the slant cultures were clearly positive while the corresponding broth cultures remained negative, or at most showed a doubtful acid change, throughout the 10-day period of observation. About one-quarter of the positive fermentations were missed entirely by the broth cultures. Except in the case of a few of the more vigorous fermenters the signs of acid production regularly appeared earlier and with more definiteness in the butt-slants than in the broth tubes.

The stab cultures seemed generally less satisfactory than either the broth or slant tubes. The superiority of the butt-slants was shown by the fact that in 12 instances fermentative changes were observable earlier and more plainly in these tubes than in the corresponding stabs, and in 7 additional sets of cultures the positive reactions on the slants were missed entirely in the soft agar tubes.

[2] This designation is used for convenience. The relation of strains of this kind to the official type species of *B. subtilis*, i.e., the Marburg type, is discussed in a subsequent paper in this series.

The greatest advantage of the agar butt-slants lies in their capacity to reveal slight and transient acid changes. Whereas in broth virtually the whole medium must be converted to an acid pH before there is a detectable indicator change, rapid or abundant growth in a small area of a slant will permit accumulation of sufficient acid at that spot to produce unmistakable evidence of fermentation. Thus, in many cases of weak fermentation the only change in the butt-slant was a more or less sharply-defined zone about the base of the slant, or in the butt, in which the indicator had changed in some degree toward the acid side.

The butt-slants also have the further advantage of showing most clearly the successive phases of pH change in the course of growth. The acid production from breakdown of the sugar in the early stages of growth is usually succeeded by a predominance of alkali production from the utilization of the peptone. This often causes parts of the acidified medium to return to the original neutral reaction in a few hours' time. In the agar butt-slants these changes are not so likely to be a source of error as in broth or semi-solid agar cultures, since the diffusion of the reaction products is less rapid and usually the reversion of a previously acidified area to neutrality begins so obviously at the periphery of the original acid zone that the changes can scarcely be misinterpreted.

The cultures of intestinal bacilli used as controls revealed their powers of fermentation equally well in all three forms of the test media. However, even with these organisms the butt-slants had at least one advantage, for they showed most clearly the striking differences in the different species as regards the speed and extent of the return of the medium to its original neutral color after the early acid change. The reappearance of a neutral reaction is apparently controlled in part by the amount of acid originally formed in any one tube, and in part by the sensitivity of the organism concerned to the accumulated acid. The cultures of *E. coli*, for example, early produced abundant acid, but the bacteria continued to grow in its presence so that the resultant alkaline products converted the whole slant back to neutrality within two or three days. On the other hand, the *E. typhosa* cultures that produced a strongly acid change within 24 hours evidently could not develop further, with the result that the acid reaction remained unchanged throughout the remaining period of observation. Among the spore-forming bacilli the actively fermenting cultures of *B. circulans* similarly showed a permanent acid change.

DIFFERENTIAL VALUE OF FERMENTATION REACTIONS IN AGAR BUTT-SLANTS

The fermentation reactions of the aerobic spore-bearing bacilli as revealed in agar butt-slants containing familiar carbohydrates have proven to be of positive, though limited, value in the differentiation of common species, especially when the capacity or lack of capacity of the organisms to store fat is also known. This latter property may be tested by staining 24 to 48-hour-old cultures on glucose or glycerol infusion agar slants with Sudan black B-safranin, as described in our earlier paper (Burdon, Stokes and Kimbrough, 1942). We have determined the characteristic fermentative powers of ten of the more prevalent varieties of spore-bearers by repeated tests in butt-slants with the 35 named stock strains available

to us and also with more than 200 strains of sporulating bacilli freshly isolated from dust and other materials. The findings are summarized in table 1.

It is evident that for purposes of differentiation the more useful carbohydrates are lactose, mannitol, and maltose. It will be seen that among the fat-positive varieties *B. megatherium* may be recognized by its fermentation of mannitol and its failure to ferment lactose. *B. circulans* is distinguishable by its relatively strong fermentation of all of the test sugars, and also by the fact that the entire butt-slant almost invariably becomes strongly acid within the first 48 hours and remains so throughout the period of observation, as mentioned above. *B. brevis* does not attack any of the carbohydrates (table 1). The remaining species cannot be definitely separated by fermentation reactions, although a weak acid change in lactose media, but not in mannitol agar, is suggestive of *B. mycoides*.

TABLE 1

Fermentation reactions of aerobic spore-bearing bacilli in agar butt-slants

FAT STAIN*	ORGANISM	GLUCOSE	LACTOSE	MANNITOL	SUCROSE	MALTOSE
Positive	B. megatherium	+++	0	++	++	++
	B. mycoides	+++	var.	0	var.	+++
	B. cereus	+++	0	0	var.	+++
	B. subtilis Michigan	+++	0	0	var.	+++
	B. circulans	++++	++	++++	++	++++
	B. brevis	0	0	0	0	0
Negative	B. subtilis Ford	+++	0	+	++	++
	B. subtilis Marburg	+	0	0	+	0
	B. vulgatus	+	0	0	+	0
	B. mesentericus	+	0	+	+	0

* Sudan black B-safranin.

+ to ++++ = fermentation with acid production, usually to the relative degree indicated.

0 = no fermentation.

Var. = variable reactions; majority show moderate amount of acid.

Among the fat-negative varieties the strains of *B. subtilis* which correspond to those we have designated by "Ford" are the only organisms that cause definite fermentation of maltose. Also, they frequently attack mannitol much more strongly than the others. These reactions are of great value since the Ford strains cannot be differentiated readily by other simple means from the Marburg strains. The relatively feeble fermentation of glucose as well as of other sugars by *B. subtilis* Marburg, and by *B. mesentericus* has been observed so constantly that it is regarded as a distinctive characteristic.

The individual strains of bacilli studied differed considerably in the vigor of fermentation. In cases of "variable" reactions (table 1) the positive fermentations were relatively weak. Freshly isolated strains usually increased their fermentative activity somewhat after two or three successive transfers in the same sugar medium. It was regularly noted, among the stock cultures and those

newly isolated as well, that a strain producing a noticeably vigorous fermentation of any one of the sugars was always relatively nore active also in the breakdown of all the other sugars it was capable of attacking.

In repeated parallel cultures individual strains of the spore-bearing bacilli showed a remarkable constancy in the degree and character of the changes produced in the indicator color; often the acid zone appeared regularly in a particular location in the butt or on the slant. However, these zones were not always duplicated by other strains of the same species, hence they cannot be relied upon for differentiation.

Since our work was completed we have noted that de Soriano (1935), in an extensive study of the whole group of aerobic spore-bearers, utilized, without special mention, a similar combination stab and slant culture with Andrade indicator, and described some species as forming acid in the stab and others on the streak. No mention is made, however, of variations in the behavior of different strains in this respect, and no comparisons are made between the results of tests in these slants and those obtained in the liquid media fermentation tubes which were also employed. The reactions reported generally agree with ours. We observed, however, a negative rather than a positive result with *Bacillus mesentericus* on maltose. In several instances we noted a positive fermentation where de Soriano reports a negative test, specifically, in the fermentation of mannitol by *Bacillus subtilis* Ford, of maltose by *Bacillus megatherium*, and of lactose and mannitol by *Bacillus circulans*.

One possible source of error in interpreting slight color changes during growth of the aerobic spore-bearers on brom-cresol-purple agar butt slants must be mentioned. The accumulation of brownish-red pigment by some of these organisms may be accompanied by changes in the indicator color which are not due to fermentation. Most conspicuous examples of this are three- to four-day-old cultures of *B. subtilis*, Marburg type, or *B. vulgatus* on lactose butt-slants, but the phenomenon is seen to a lesser degree with other organisms and other sugars. The original purple color of the medium about the growth changes to a purplish-red, a color indistinguishable from that which may result from slight acid formation. The reddish zone appears along the slant only, however, and extends just a few millimeters into the medium below the surface growth. It is not a sign of fermentation, for it is found associated always with a pigmented growth, proportional in its extent to the amount of visible pigment, and it is absent in non-pigmented cultures of the same species.

For correct interpretation of such changes, as well as of minimal fermentation reactions, it is necessary, of course, to compare the test cultures directly with tubes of uninoculated medium of the same lot and to make frequent inspections of the fermentation tubes during incubation for several days.

SUMMARY

Agar slants with deep butts, inoculated into the butt as well as over the slant, were found superior to broth (Durham fermentation tubes) or soft agar stabs for fermentation tests with the common species of aerobic spore-forming bacilli.

The butt-slants, made with 1.5 per cent agar and containing brom-cresol-purple as indicator, proved more sensitive than the other types of media for the detection of slight acid production and often gave positive evidence of fermentation when the broth or stab cultures remained negative. When closely observed over a considerable period of time, the butt-slants also revealed more clearly other properties which were characteristic of individual strains of these bacilli, such as the relative vigor of fermentation, the speed and extent of reversion to a neutral or alkaline reaction after the initial acid change, and the appearance of zones of acid reaction in particular locations in the medium.

The fermentation reactions of the ten principal varieties of aerobic spore-bearers, as determined by cultures in brom-cresol-purple agar butt-slants containing familiar carbohydrates, were found to be of definite, though limited, differential value, especially when the capacity or lack of capacity of the organisms to store fat is also known. Among the fat-positive varieties, *Bacillus megatherium*, *B. circulans*, and *B. brevis* are readily recognized by distinctive fermentations in lactose, maltose, and mannitol. Among the fat-negative species, reactions in the same sugars are of special aid in distinguishing *Bacillus subtilis* Ford from the Marburg type of *B. subtilis*.

REFERENCES

Bergey, D. H., and others 1939 Bergey's Manual of Determinative Bacteriology, 5th Ed., p. 640. Williams & Wilkins Co., Baltimore.

Burdon, K. L., Stokes, J. C., and Kimbrough, C. E. 1942 Studies of the common aerobic spore-forming bacilli. I. Staining for fat with Sudan black B-Safranin. J. Bact., **43**, 717–724.

Conn, H. J , and Hucker, G. J. 1920 The use of agar slants in detecting fermentation. J. Bact., **5**, 433.

Ford, W. W. 1927 Textbook of Bacteriology, p. 659. Saunders Co., Philadelphia.

Lamanna, C. 1940 The taxonomy of the genus *Bacillus*. II. Differentiation of small-celled species by means of spore antigens. J. Infectious Diseases, **67**, 193–204.

Lamanna, C. 1940 The taxonomy of the genus *Bacillus*. III. Differentiation of the large-celled species by means of spore antigens. J. Infectious Diseases, **67**, 205–212.

de Soriano, A. M. 1935 Estudio sistemático de algunas bacterias esporuladas aerobias. Rev. inst. bacteriol. dept. nac. hig. (Buenos Aires), **6**, 507–642.

Topley, W. W. C., and Wilson, G. S. 1936 Principles of Bacteriology and Immunity, 2nd Ed., p. 654. William Wood & Co., Baltimore.

BACTERIA ATTACKING PETROLEUM AND OIL FRACTIONS

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Although a number of workers have reported that certain bacteria and molds are capable of attacking hydrocarbons and mineral oils it is still not generally recognized how widespread is the occurrence of these organisms nor how great a variety of compounds of this nature may be attacked. Since Söhngen (1906) showed that some microorganisms possess the ability to utilize methane and Rahn (1906) described molds capable of attacking paraffin, there have appeared about thirty papers on the subject (cf. Hessel, 1924, and Tausson, 1929). Various gram-negative rods, particularly *Pseudomonas*, mycobacteria and micrococci have been described as attacking petroleum but there has been little effort made to compare organisms having this ability with bacteria that obtain their growth energy from the ordinary sources. Recently Bushnell and Haas (1941) have studied hydrocarbon utilization by various organisms and have found that *Pseudomonas* strains are most active while certain species of micrococci and corynebacteria are also able to assimilate these compounds.

The conditions necessary for attack on oils by microorganisms have been summarized by Tausson (1928) as follows: (1) presence of water with mineral salts; (2) a nitrogen source, such as the ammonium or nitrate ion; (3) free access of oxygen; (4) a neutral reaction and a buffer such as $CaCO_3$ to maintain it.

However, there has been little attention given as to what hydrocarbons are most subject to attack and the mechanism of their breakdown, although Tausson and co-workers (1934) have shown that some acids and unsaturation are produced in the bacterial dissimilation of crude and lubricating oils.

The purpose of this work was to determine how wide a range of petroleum fractions could be readily attacked, to attempt to find which of several representative petroleum fractions were most subject to attack, and to isolate and characterize a number of organisms able to develop on a hydrocarbon medium.

DEVELOPMENT OF CULTURES ON OIL

Erlenmeyer flasks were prepared containing 0.5 g. oil, 50 ml. H_2O, 0.25 g. $CaCO_3$, 0.25 per cent NH_4NO_3, 0.1 per cent Na_2HPO_4, 0.05 per cent KH_2PO_4, 0.05 per cent $MgSO_4$, 0.02 per cent $MnCl_2$, and traces of Ca, Fe and Zn. The flasks were inoculated with one gram of garden soil, incubated for a period of from 10 to 20 days and shaken twice daily. When considerable decomposition appeared as indicated by the emulsification of the oil and increased turbidity of the medium, 1 ml. of the mixture was transferred to another flask containing all the above ingredients except soil. After 2 to 3 transfers the breakdown of the oil proceeded faster and the period of incubation was shortened accordingly. Flasks were incubated at 20°, room temperature (23–26°), 30°, and 37°C.

After at least 6 successive transfers from the original flask containing soil inoculum, bacterial counts were made on the medium by plating on standard nutrient agar. A mineral-salt oil agar was also used, but it was observed that nutrient agar gave slightly higher counts. Furthermore, in all cases tested the organisms which appeared on the mineral-salt oil medium grew when transplanted to the nutrient agar. As continued cultivation on nutrient agar caused a marked decrease in the ability of the cultures to attack hydrocarbons, cultures were picked from the plates in proportion to the numbers present and kept on the mineral-salt oil medium.

The materials used in this study included several crude oils, heavy oil residues such as petrolatum (paraffinic) and asphaltic tar (aromatic) and the various oils given in table 1. The filtered 185 Pennsylvania neutral was a conventionally-refined 10-W grade of motor oil. This oil is typical of light oils or neutrals made

TABLE 1

Physical properties of oil fractions studied

OIL NO.	DESCRIPTION	CENTISTOKES VISCOSITY		ESTIMATED COMPOSITION*			CALC.† MOL. WT.	EST. AVE. NO. RINGS PER MOLECULE
		At 210°F.	At 100°F.	% A	% N	% P		
1	Filtered 185 Pa. Neutral	5 93	39.86	9	15	76	407	1.7
2	Portion of (1) Distilled	3.56	17 76	9	16	75	328	1.4
3	Portion of (1) Distilled	5 91	40 37	8	16	76	402	1.7
4	Portion of (1) Distilled	9.66	86.03	9	14	77	504	2.0
5	Portion of (1) Extracted	8 39	120 9	29	13	58	347	2.4
6	Portion of (1) Extracted	4.86	28 19	0	24	76	377	1.5
7	Portion of (1) Extracted	7 00	44 69	0	17	83	459	1 3
8	Penna. Bright Stock	32.91	540	9	13	78	740	2 9
9	Final Raffinate from (8) Extracted	103.8	2321	0	(14)	(86)	1194	(3.1)
10	Portion of Bosco Neutral Extracted	11.1	350.8	54	19	27	270	3.2

* Values obtained using method of Vlugter, et al. (1935). % A = % aromatics; % N = % naphthenes; % P = % paraffins.

† Obtained using a modified Keith and Roess method (1937).

in the Pennsylvania area, and is a relatively homogeneous mixture of hydrocarbons. The distillation fractions of this oil, designated as oils 2, 3, and 4 in table 1, were prepared by high vacuum fractional distillation and differ from each other essentially in molecular weight. Each oil is a narrow boiling fraction with a very small molecular weight range.

Oils 5, 6, and 7 of table 1 were prepared by solvent-extraction of oil with acetone (Hersh 1938). Thus, these fractions represent hydrocarbons differing essentially in molecular structure, whereas those prepared by distillation represent fractions differing essentially in molecular size or weight.

Oil 8 is a typical heavy grade oil with a higher molecular weight and more heterogeneous composition than oil 1. Oil 9 resulted from exhaustive acetone extraction of oil 8 and may be considered to be a very high molecular weight, highly paraffinic hydrocarbon mixture.

Oil 10, an aromatic extract from a Louisiana light distillate oil, represents a relatively low molecular weight aromatic-type hydrocarbon oil.

Mixed cultures were developed from soil capable of attacking each oil or oil fraction studied as well as the crudes, petrolatum and asphalt. After several transfers the medium weight and crude oils were found to be quite rapidly broken down and usually completely emulsified in 3 to 5 days at room temperature. The heavier oils were more slowly dissimilated, the emulsification process requiring as long as 3 weeks. Bacterial counts were made on the various fermentations after 6 to 10 consecutive transfers. Petrolatum and asphalt supported the growth of large numbers of organisms concentrated at the oil-water interface of the separate globules, but owing to the difficulties of dispersion, no counts were made on these materials.

TABLE 2

Fermentation of oils at room temperature

Bacterial plate counts in millions per ml. of one per cent oil dispersed in a mineral salt medium

TYPE OF OIL	TIME OF FERMENTATION IN DAYS							
	0	1	2	4	6	8	10	14
Penna Crude	13	65	300	380	220	121		
Mich. Crude	8.3	44	420	680	780	290		
Oil No. 1	0.6	35	640	510	380	390		25
Oil No. 2	1.3	65	620	970	720	740		90
Oil No. 3	0.6	88	350	400	540	670		104
Oil No. 4	1.0	47	180	165	210	370		99
Oil No. 5	0.6	121	440	310	300	280		71
Oil No. 6	0.1	270	390	690	140	610		270
Oil No. 7	0.3	14	510	1470	1150	880		39
Oil No. 8	1.2		90	240	91		240	180
Oil No. 9	0.4		6	12	21		51	36
Oil No. 10	1.1		55	87	250		108	157

Typical counts of the mixed flora found in the breakdown of crude oil and certain fractions are shown in table 2. In the majority of cases the peak in bacterial flora occurred between the third and sixth day of fermentation. At this time there was usually one type of colony predominating in each type of oil. All the organisms examined from the various oils at the different temperatures appeared to be short, motile, gram-negative rods. In the crude oils *Pseudomonas* seemed to be the outstanding type. White opaque colonies appeared most frequently in the light oils. White and mucoid colonies were predominant in many of the heavy oils. In most cases pigmented forms other than *Pseudomonas* did not appear until after a peak in total count was reached. There was very little indication of the specificity of a certain organism for a particular type of oil. However, if a mixed culture from a rapidly fermenting oil of highly aromatic

nature was transferred to a paraffinic type (or *vice versa*) the growth lagged for several days. The culture was usually able to adapt itself to its environment and become active in the new oil within one transfer.

It was apparent that the lighter weight oils were more easily attacked than the heavier fractions. This is evident from the counts given in table 2, if counts of oils 2 and 4 are compared, or of oils 2 and 8, or oils 8 and 9. In the fermentation of mixtures containing as much as 5 to 10 per cent of Pennsylvania 185 neutral oil measurements of the unfermented oil showed an increase in viscosity, indicating a preference of the organisms for the lighter fractions. Also, it could be readily observed that the light oils disappeared from the surface of the medium much more quickly than the heavy ones. The crudes supported growth in a degree comparable to the light oils and were subject to rapid emulsification although globules of the heavy fractions could still be discerned on the surface after two weeks' fermentation.

The bacterial counts also indicate that the paraffinic oils were attacked more rapidly than the aromatic types as can be seen by comparing aromatic oil 5 with paraffinic oil 7. Oil 6, which contains a relatively high per cent of naphthene compounds occupied an intermediate position with respect to growth supported. The slower attack of aromatic oils can also be shown by comparing oil 10 (strongly aromatic) with oil 7 or the light neutrals 1, 2, and 3. Although oil 10 is more viscous than the light oils it has a smaller average molecular weight.

MANOMETRIC STUDIES

Several representative oils were chosen for determination of the oxygen required for their dissimilation by mixed cultures. The studies were carried out with a Warburg manometric apparatus immersed in a constant temperature bath and shaken continuously by motor. The technique and apparatus used was similar to that described by Dixon (1934). The cultures were inoculated and cultivated in standard manometric flasks and respiration measured over a period of several days at 30°C. In each flask was placed 3.8 ml. of mineral salt solution, 50 mg. of the desired oil and 0.2 ml. of the corresponding mixed culture inoculum. The mineral salt solution was of the same composition as that described previously except that $(NH_4)_2SO_4$ was substituted for NH_4NO_3. Readings were taken twice daily for intervals of two hours, these figures were plotted and from the area subtended by the curve the total respiration during the entire time was calculated.

When the fermentations were carried out for longer than 7 days, a large fluctuation in results was noted. Allowing for small errors in technique and the relatively large variation to be expected from mixed culture studies, the results obtained during the first 5 days of incubation were fairly consistent. In practically every case the peak of respiratory activity came in this interval. However, it was noted that the actual amounts of oxygen consumed and carbon dioxide liberated varied according to the rate of shaking, the temperature of incubation and the age of the inoculum. Some typical results are shown in table 3. About 10 ml. of oxygen were taken up in the fermentation of oils 1

and 7 containing predominantly paraffin hydrocarbons. In oil 5 (aromatic) the uptake was slower. The breakdown of oil 8, which has an average molecular weight of nearly two times the light oils and is more viscous, consumed only about half as much oxygen during the longer time interval. Oil 9 which is still heavier and composed of larger molecules had still less oxygen uptake.

As a check on the respiration to be expected from the cells alone, a control is shown based on 0.2 ml. of inoculum from oil fermentation suspended in buffer with no oil added. The amount of respiration is negligible in comparison with the growing culture.

TABLE 3

Oxygen uptake and carbon dioxide evolved in oil dissimilation

OIL	TRIAL	TIME OF INCUBATION DAYS	OXYGEN UPTAKE	CO_2 EVOLVED	CO_2 (CORRECTED)	RATIO CO_2/O_2
			ml.	*ml.*	*ml.*	
1	1	6	11.80	7.81	7.42	0.63
	2	5	11.06	7 70	7 31	0 66
	3	5	9 77	6.62	6 29	0 64
5	1	5	6.82	4.52	4.29	0 63
	2	5	7.32	5.14	4.88	0.67
	3	5	7.21	4.77	4.53	0 63
7	1	5	10.89	7.81	7 42	0.68
	2	5	11.23	8.14	7 74	0 69
	3	5	9 77	6.94	6.59	0.67
8	1	8	6.17	2.92	2.77	0 45
	2	8	7.50	3.63	3.45	0 46
	3	8	4.23	2.09	1.99	0 47
9	1	13	2.17	0.42	0.40	0 18
	2	13	3.08	0.24	0 23	0.07
Control (1)	1	5	0.24	0.16	0.15	
	2	5	0 17	0.11	0 11	

The carbon dioxide values are subject to an error as a small amount of gas would originate from the $CaCO_3$ buffer, not only by the action of organic acids that may have been formed during fermentation but also from free sulfuric acid produced by the utilization of nitrogen from the $(NH_4)_2SO_4$. The latter can be roughly estimated at between 0.08 and 0.1 ml. CO_2 for 4 ml. of medium on an active culture producing nearly a gram of moist cells per liter, assuming that the cells contained 2.5 per cent nitrogen on a wet basis. As large scale fermentations carried out under similar conditions indicate that usually about 80 per cent of the original oil can be recovered, only about 0.20 to 0.30 ml. of CO_2 could be liberated by mono-carboxylic acids formed from 0.050 g. of the oil having a ml. wt. in the range of 400. The total correction amounts to an approximate reduc-

tion of only 5 per cent for the CO_2 evolution of the light oils. The heavy oils have been corrected accordingly.

The CO_2/O_2 ratios using the corrected values are subject to the errors present in the CO_2 determination but show fairly consistent values for the different oils. It is noteworthy that in the case of the light oils, the ratios are high for incomplete oxidation of the hydrocarbons. The theoretical respiratory quotient for complete oxidation of a long-chain paraffin hydrocarbon with the formula $CH_3(CH_2)_nCH_3$ is approximately 0.67.

$$(CH_2)_n + 1.5nO_2 \rightarrow nCO_2 + nH_2O$$
$$CO_2/O_2 = n/1.5 \times n = 0.67$$

The respiratory quotients of the light oils are in the neighborhood of 0.65.

If the hydrocarbon molecules were oxidized without decarboxylation and considerable loss of CO_2 the gas ratios should be much lower. The results indicate that a large percentage of the molecules attacked were completely oxidized to CO_2.

The fermentation of the heavier oils containing longer molecules not only did not produce as much CO_2 but furthermore gave a much lower CO_2 to O_2 ratio. In the case of oil 9 the CO_2 evolution was practically neglible, indicating a much less complete oxidation of the heavy oils.

PURE CULTURE STUDIES

Organisms isolated from the mixed cultures by plating on either standard nutrient or mineral-salt oil agar were kept in a mineral salt broth to which had been added a few drops of sterile oil corresponding to that from which they were isolated. The reactions of these organisms were determined on litmus milk, gelatin and glucose, sucrose, lactose, arabinose, salicin, mannitol and nitrate broths. Tests made for indole, acetylmethylcarbinol, and the methyl-red reaction were negative in all cases. An oil mineral-salt broth containing bromthymol-blue to indicate acid production and without $CaCO_3$ was also used to determine whether or not the cultures were specific for any particular type of oil or oil fraction. The mineral-salt broth was sterilized in tubes and a drop of the sterile oil to be tested was added aseptically at the time of inoculation.

A study of 250 representative cultures gave no evidence that a particular morphological type or biochemical reaction could be correlated with any type of oil or oil fraction. Furthermore, with the exception of certain pigmented forms, there was no correlation between colony morphology and reactions in the ordinary laboratory media. There was considerable difference in the response of cultures to laboratory media when they were first picked from an actively growing hydrocarbon-splitting culture, compared to the same cultures that had been carried on nutrient agar for a number of transfers. Generally it was noted that organisms capable of rapid growth in an oil medium grew luxuriantly on nutrient agar. Many were quite mucoid and formed large glistening gelatinous colonies. Observations of cultures picked from a single colony on agar gave evidence that the organisms changed in their biochemical reactions as well as in their morphology when placed in a hydrocarbon environment.

Although the organisms found represent a variety of types, there are few outstanding lines of division for classification into groups. When pure cultures were grown in the various oil media, it was found that generally no organism was limited to the attack on one type of oil. Usually it would attack many different oils with about equal facility. Oils 9 and 10 were particularly resistent to breakdown, however, due to their high viscosities.

A tentative grouping of the organisms found, permits separation into three main groups based principally on colony morphology on nutrient agar and action on glucose broth, litmus milk, gelatin and oils. A summary of these data is given in table 4. The predominant organisms, group I, were members of *Pseudomonas* and were mostly of one characteristic type that liquefied gelatin, reduced and peptonized litmus milk, formed acid in glucose broth, and produced a neutral

TABLE 4

Organisms isolated from oil fermentation

GROUP	MORPHOLOGY ON AGAR	NO. ISOLATED	GLUCOSE	LITMUS MILK	NITRATE RED'N	GELATIN LIQUEFACTION	OIL MEDIA	PROBABLE GENUS
I	Green, flat, spreading	63	A	RP	+	Stratiform	F	Pseudomonas (aeruginosa)
IIa	White, convex, mucoid	46	A	A or –	±	–	A	Achromobacter
IIb	White, convex, mucoid	26	A	R	±	Slow or –	F	Achromobacter
IIIa	White, mucoid, sometimes brownish	28	–	RP	±	–	A	Alcaligenes (radiobacter)
IIIb	White, mucoid, sometimes brownish	52	–	Al	–	–	F	Alcaligenes
Misc.	Reds, yellows, whites, etc.	35						Mixed

reaction in oils. The reactions of this group were quite consistent. Flagella stains and serological tests on certain typical cultures have shown them to possess one long polar flagellum and to be apparently identical with a stock culture of *Pseudomonas aeruginosa*. This organism was likewise found to be the chief species active on hydrocarbons by Bushnell and Haas (1941).

The other groups did not exhibit such clear cut characteristics, although all were motile, gram-negative rods. Most of them were white, mucoid types, many of them possessing a buff or brownish sheen. The organisms that usually gave an acid reaction in glucose and acid or reduction in litmus milk have been placed in group II. This group has been subdivided on the basis of the ability of many of the organisms to form acid in oils. However, there is evidence that this differential characteristic is no more constant among these bacteria than

action on gelatin or nitrate. Generally the members of group II that were active producers of acid in oil produced acid in litmus milk. The organisms of group II should probably be classed as *Achromobacter* although there is no species described in Bergey's Manual (1939) that corresponds to all their reactions. In many ways the members of this group are similar to *Achromobacter arvilla* and *A. sapolium* described by Gray and Thornton (1928) and listed as *Pseudomonas* by Bergey's Manual. There is little doubt that these authors were dealing with the same type of organisms encountered in the present study.

The third group of organisms displayed no marked action on sugars, and usually produced an alkaline reaction in litmus milk. In many respects these bacteria resemble the common soil forms that have been variously described as *Radiobacter* or *Alcaligenes radiobacter*. Group III was likewise divided into parts "a" and "b" on the formation of acid in oils. However, as in group II the characteristics of these organisms were not clear cut and considerable variation was noted among the reactions of the same culture carried on different media.

A few miscellaneous organisms comprising some red, yellow, white, and fluorescent types have not been grouped as there were not sufficient numbers of any one type to compare with the ones that have been discussed. The red and yellow colonies tended to disappear from the mixed cultures as transfers were continued.

DISCUSSION

It is evident from the studies presented here together with those of other investigators, that under favorable conditions microorganisms can be found that are capable of attacking practically any hydrocarbon from methane up to the heaviest paraffinic or asphaltic residues. However, the heavier oils become more difficult to attack as the viscosity and molecular weight increase. This is due in part to the fact that the more viscous oils are harder to disperse in a liquid medium and hence there is less surface exposed to the growth of microorganisms. But the difficulty of attack is probably also attributable to the larger molecule. Strawinski and Stone (1940) have found that compounds in the range of 10 to 16 carbon atoms are attacked more readily than those of smaller molecular weight. Observations by the authors as well as Bushnell and Haas (1941) on the fermentation of gasoline and kerosene indicate that, although both substances are quickly attacked, the kerosene is more subject to break down than the gasoline with bacterial counts up to a billion per ml. or comparable to counts observed in light oils. From these results, it is evident that in oils of a predominantly paraffinic nature the fractions from kerosene up to medium weight lubricating oils include the range most easily attacked by bacteria.

Both bacterial counts and manometric studies indicate that paraffinic oils are more easily broken down than corresponding oils of aromatic nature. However, it must be emphasized that the predominantly aromatic fractions proved to be very acceptable carbon and energy sources. The most unexpected observation in this connection was the apparent lack of specificity of one culture for any certain type of oil. Even when such studies are extended to pure hydrocarbons

it is possible to find that an organism which is adapted to grow on a purely paraffinic source such as cetane will begin to grow immediately when transferred to a strictly aromatic compound such as naphthalene.

The organisms capable of attacking oils appear to be present wherever samples were taken. Only gram-negative rods were found, whether the enrichment cultures were incubated at 20°, 30°, or 37°C. These findings are not entirely in accord with Söhngen (1913) who observed mycobacteria at 37°C. nor with Bushnell and Haas (1941). One reason for the absence of acid-fast bacteria in the present work is that the cultures were transferred at intervals of 7 to 14 days and probably there was not sufficient time for the development of these slower-growing organisms.

It is evident, particularly among the non-pigmented forms, that there is no sharp line of demarcation between the different groups. The adaptability of these bacteria is further realized when it is considered that in most soil samples there is little chance of long chain hydrocarbons having been present for unnumbered bacterial generations. It is obvious that there is no specialized group of organisms here but that we are dealing with the common soil forms which possess the ability to adapt themselves to an infinite variety of organic compounds.

SUMMARY

1. Cultures capable of attacking crude oil, lubricating oils, vaseline, asphalt, and all other petroleum fractions used were obtained from garden soil.

2. It was found that the light to medium-weight fractions are more subject to attack than the heavy viscous portions and that the paraffinic fractions are more readily broken down than the aromatic types.

3. The breakdown of oil is an oxidative change characterized by a high bacterial count, emulsification and sometimes a decrease in pH.

4. There was much less oxygen uptake in fermentations of heavy oils compared to similar lighter fractions. Likewise aromatic fractions utilized less oxygen than the paraffinic types.

5. The CO_2 to O_2 ratio for the dissimilation of light oils is in the neighborhood of 0.65. In heavy oils the ratio drops to a much lower figure.

6. The organisms were all motile gram-negative rods including *Pseudomonas*, and many white-mucoid types. They were obtained from all soil samples tested and appear to be of common occurrence.

7. The cultures did not exhibit a specific ability to attack one type of oil but rather a capacity to adapt themselves, according to conditions, to attack the particular oil that was present.

REFERENCES

Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1939 Bergey's Manual of Determinative Bacteriology. Fifth edition. Williams & Wilkins, Baltimore.

Bushnell, L. D., and Haas, H. F. 1941 The utilization of certain hydrocarbons by microorganisms. J. Bact., **41**, 653–673.

Dixon 1934 Manometric Methods. Chap. VI. Cambridge University Press.

Gray, P. H. H., and Thornton, H. G. 1928 Soil bacteria that decompose certain aromatic compounds. Zentr. Bakt., Parasitenk., II, **73**, 74–96.

Hersh, R. E., Varteressian, K. A., Rusk, R. A., and Fenske, M. R. 1938 Discontinuous fractional extraction apparatus utilizing reflux. Ind. Eng. Chem., Anal. Ed., **10**, 86–91.

Hessel, F. A. 1924 Action des bactéries sur les hydrocarbures et en particulierer sur les pètroles. Mat. grasses, **16**, 6936–40.

Keith, J. R., and Roess, L. C. 1937 Molecular weights of viscous petroleum fractions. J. Ind. Eng. Chem., **29**, 460–4.

Rahn, O. 1906 Ein Paraffin zersetzender Schimmelpilz. Zentr. Bakt., Parasitenk., II, **16**, 382–4.

Söhngen, N. L. 1906 Ueber Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. Zentr. Bakt., Parasitenk., II, **15**, 513–7.

Söhngen, N. L. 1913 Benzin, Petroleum, Paraffinol, und Paraffin als Kohlenstoff-und Energiequelle fur Mikroben. Zentr. Bakt., Parasitenk., II, **37**, 595–609.

Strawinski, R. J., and Stone, R. W. 1940 The utilization of hydrocarbons by bacteria. J. Bact., **40**, 461.

Tausson, W. O. 1928 Bacterial oxidation of crude oils. Neftyanoe Khoz., **14**, 220–30.

Tausson, W. O. 1929 Über die Oxydation der Benzolkkohlen-wasserstoffe durch Bakterien. Planta (Z. wiss. Biol. Abt. E.), **7**, 736–57.

Tausson, W. O. 1934 The general trend of the process of oxidation of oil by bacteria. Microbiology (USSR), **3**, 79–87.

Vlugter, J. W., Waterman, H. I., and van Westen, H. A. 1935 Improved methods of examining mineral oils, especially the high boiling components. J. Inst. Petroleum Tech., **21**, 661–76.

THE UTILIZATION OF AMINO ACIDS AND RELATED COMPOUNDS BY CLOSTRIDIUM TETANI[1]

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Studies on the metabolism of *Clostridium tetani* have been confined to the general changes during growth in various media. Woods and Clifton, 1937, and Barker, 1937, reported that *Clostridium tetanomorphum*, an anaerobe apparently closely related to *C. tetani*, obtains its energy by the fermentation of certain amino acids, while *Clostridium sporogenes* (Stickland, 1934–35) and *C. botulinum* (Clifton, 1940) utilize amino acids primarily by means of the "Stickland reaction", a coupled oxidation-reduction between pairs of different amino acids. These studies on the utilization of amino acids and of possible intermediate products by the pathogenic anaerobes (and closely related forms) have been extended to include the metabolic activities of *C. tetani*.

The methods employed were essentially those previously described (Clifton, 1940). The strain of *C. tetani* (E-35), obtained from Dr. Ivan Hall's collection (No. 130A), is an active toxin producer.

RESULTS

The organisms were cultivated in broth containing 1 per cent glucose and 0.1 per cent yeast extract (Difco), growth appearing to be more rapid in the presence of glucose although no evidence was obtained indicating that glucose was utilized. After 7 days at 37°C. the main products were alcohol, volatile and non-volatile acids, ammonia, carbon dioxide and small amounts of hydrogen. Duclaux distillations of the bichromate oxidation products of the alcohol suggested a mixture of ethyl and butyl alcohols. The actual formation of butyl alcohol and the ratio of the alcohols were not determined. Acetic and butyric acids were found in a ratio of approximately 2 to 1. The non-volatile, ether-soluble acid appeared to be approximately 3 parts of lactic to 1 part of an acid tentatively identified as malic.

Carbon dioxide and hydrogen production by washed suspensions of 20-hour cultures of *C. tetani* in the presence of the more common amino acids, glucose, glycerol, and certain non-amino acids was determined at 37°C. by the Warburg technic. The contents of the Warburg vessels were also analyzed for ammonia when amino acids were employed as the substrate. The amino acids attacked were fermented directly, pairs of amino acids not being required as with *C. sporogenes* or *C. botulinum*. The amounts of hydrogen produced were in most instances not markedly greater than those produced by the control suspensions. Hydrogen production varied to some extent with different suspensions and substrates but never exceeded 2–5 per cent of the total gas produced. The rate of

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hydrogen production was most rapid immediately after the substrate was added and decreased to the level of the control suspension more rapidly than the rate of carbon dioxide production. Hydrogen production was decreased when hydrogen was substituted for nitrogen as the gas phase. Some evidence, as yet inconclusive, was obtained of a slight absorption of hydrogen with certain substrates. The studies on hydrogen production by *C. tetani* are incomplete but it is apparent that this organism is a much less active hydrogen producer than *C. tetanomorphum* (Woods and Clifton, 1937). The results of tests for carbon dioxide and ammonia production were essentially the same with either nitrogen or hydrogen as the gas phase and are summarized in table 1.

The substances attacked by *C. tetani* are similar to those reported as utilized by *C. tetanomorphum* (Woods and Clifton, 1937) except that glycerol, glucose and malic acid are not attacked. The rate of carbon dioxide production from

TABLE 1

Carbon dioxide and ammonia production from various substrates (M/100) by washed suspensions of C. tetani in M/15 phosphate buffer of pH 7.0

	AMINO ACIDS			ACIDS		
	CO_2	Q_{CO_2}	NH_3		CO_2	Q_{CO_2}
Glycine	−	−	−	Formic	−	−
d-alanine	−	−	−	Acetic	−	−
Glycine + d-alanine	−	−	−	Butyric	−	−
				Lactic	−	−
dl-serine	+	30–40	+	Pyruvic	+	40–50
l-proline	±	−	−	Maleic	−	−
d-glutamic	+	40–50	+	Malic	−	−
l-aspartic	+	5–10	+	Glycollic	−	−
Glycerol	−	−	−	Fumaric	+	30–40
Glucose	−	−	−	Succinic	−	−

proline was not much greater than that observed with the control suspension and may be due to the presence of traces of a utilizable impurity.

The influence of pH on the rate of carbon dioxide production from the substrates utilized by *C. tetani* was determined over a pH range of 5.6 to 7.5. The rate of carbon dioxide production from a given substrate was quite constant over a pH range of 6.0 to 7.2, no marked pH optimum being observed with any of the substrates. Above 7.2 and below pH 6.0 the rates tended to decrease. A pH of 6.4 was employed in the majority of studies in order to reduce as far as possible the bound CO_2. In the few tests made at pH 7.2, the rates and products of metabolism were essentially the same as at pH 6.4.

The various substrates were studied in more detail in semi-macro Warburg studies and on a macro scale in experiments of 20-hour duration. Essentially similar results were obtained, and since the analyses are more accurate in the larger scale experiments the results of the latter are presented. Respiration of the control suspensions was negligible in these studies and therefore controls

TABLE 2

Fermentation balances with various substrates following 20 hours' incubation with washed suspensions of C. tetani

	PRODUCTS								
	CO_2	NH_3	Acetic	Butyric	Lactic	Malic	Alcohol*	Total	Theoretical
Per millimol of pyruvic acid									
Mols/mol..	0.9		0.18	0.36	0.10				
Mgm... . .	39.6		10.8	31.7	9.0			91.1	88.0
Mgm. C	10.8		4.3	17 3	3.6			36.0	36.0
Mgm. H. .			0.7	2.9	0.6			4.2	4.0
Mgm. O	28.8		5.8	11.5	4.8			50 9	48.0
	Available —H (Barker, 1936). Found 9.8. Theoretical 10.0								
Per 2.5 millimols of fumaric acid									
Mols/mol.... ..	1.3		0.1	0 1	0 05	0.19	0.35		
Mgm. .	138.2		13 8	20.2	10.8	64.3	39.9	287.2	290.0
Mgm. C	37.7		5.5	11.0	4.3	23.0	20.9	102.4	120.0
Mgm. H .			0.9	1.8	0.7	2 9	5 2	11.5	10 0
Mgm. O	100.5		7 4	7 4	5.8	38.4	13.9	173.4	160.0
	Available —H. Found 24.1. Theoretical 30								
Per 2.87 millimols of serine on basis of NH_3 production									
Mols/mol... .	1 0	1 0	0.23	0.23			0.24		
Mgm.. .	127.6	48 8	40.2	59.0			32 2	307 8	301 4
Mgm. C .	34 8		16.1	32.2			16.8	99.9	103 3
Mgm. H . .		8 6	2 7	5.4			4.2	20 9	20 1
Mgm. O . .	92 8		21.4	21.4			11 2	146 8	133 8
	Available —H. Found 35.8. Theoretical 37 3								
Per 0.98 millimols of glutamic acid on basis of NH_3 production									
Mols/mol. .	0.94	1.0	0.90	0.45	trace				
Mgm..........	41.4	16.6	54.0	39.6				151.6	147.1
Mgm. C......	11.3		21.6	21.6				54 5	60 0
Mgm. H		2 9	3.6	3 6				10.1	9 0
Mgm. O. .. .	30.1		28.8	14.4				73 3	64 0
	Available —H. Found 19.1. Theoretical 20 6								
Per 0.92 millimols of aspartic acid on basis of NH_3 production									
Mols/mol. ..	1.05	1.0	0.18†		0.55†		0.3		
Mgm... .	46.2	12.9	10.9		49.5		13 8	133.3	122.4
Mgm. C.. . .	12.6		4.4		19.8		7.2	44.0	44 2
Mgm. H .		2.8	0 7		3.3		1 8	8.6	6.5
Mgm. O . ..	33.6		5.8		26.4		4.8	70.6	58 9
	Available —H. Found 14.4. Theoretical 13.8								

* Reported as ethyl alcohol.

† Reported as acetic and lactic acids, butyric and malic acids also probably found.

were generally omitted. All studies reported were carried out in M/15 phosphate buffer of pH 6.4 and at 37°C. with hydrogen as the gas phase.

Pyruvic acid was readily utilized by *C. tetani*, the main products being carbon dioxide and lactic, acetic and butyric acids. Duclaux distillation indicated a ratio of approximately 1 part of acetic to 2 parts of butyric acid. The same products were obtained with the other substrates tested, the ratio of acetic to butyric acid varying with the substrate. In addition an alcohol, or alcohols, was produced from fumaric and aspartic acids and from serine. Duclaux distillations of the bichromate oxidation products suggested the production of some butyl alcohol together with ethyl alcohol. The results of these distillations were only qualitative in character and the alcohol will be reported *as ethyl alcohol.*

A portion of the fumaric acid appeared to be converted to malic acid which is not utilized by *C. tetani.* The evidence for the formation of malic acid rests on the observations that the non-volatile, ether-soluble, acidic products were readily oxidized by potassium permanganate in acid solution at 37°C. Since lactic acid could be readily determined, the difference between the lactic and the total non-volatile acid was considered to be malic acid. The quantitative carbon dioxide production on permanganate oxidation (method of S. F. Carson, personal communication), 1 mol of carbon dioxide being produced per mol of lactic acid and 3 mols per mol of malic acid oxidized, agreed closely with that postulated from the results obtained by the former method. In addition, a small amount of a silver salt was isolated, the silver content of which agreed well with the theoretical value for silver malate (found 62.6, theory 62.0 per cent). Succinic acid is not attacked by *C. tetani* and could not be detected as a product of fumarate utilization. Typical results of the fermentation studies are presented in table 2. The results reported for aspartic acid are tentative since it is attacked at the slowest rate of any of the substrates utilized by *C. tetani.* The products from aspartic acid are reported as acetic and lactic acids and ethyl alcohol, but it must be borne in mind that butyric and malic acids and possibly butyl alcohol may also be produced. The values for available $-H$, with the exception of that found for fumaric acid utilization, are in good agreement with the theoretical.

SUMMARY

Glutamic and aspartic acids and serine are decomposed by suspensions of *Clostridium tetani*, carbon dioxide, ammonia, and acetic and butyric acids being the main products of decomposition. Appreciable amounts of lactic acid and alcohol are also produced from aspartic acid. The "Stickland reaction" is not involved in the metabolic activities of *C. tetani.*

Essentially the same products are produced during the dissimilation of pyruvic and fumaric acids, malic acid being an additional product in the case of fumarate utilization. Glucose does not appear to be attacked by *C. tetani.*

REFERENCES

BARKER, H. A. 1936 On the fermentation of some C_4-dibasic acids by *Aerobacter aerogenes.* Koninkl. Akad. Wetenschappen Amsterdam, **39**, 674–683.

Barker, H. A. 1937 On the fermentation of glutamic acid. Encymologia, **2**, 175–182.

Clifton, C. E. 1940 The utilization of amino acids and of glucose by *Clostridium botulinum*. J. Bact., **39**, 485–497.

Stickland, L. H. 1934 The chemical reactions by which *Cl. sporogenes* obtains its energy. Biochem. J., **28**, 1746–1759.

Stickland, L. H. 1935 The reduction of proline by *Cl. sporogenes*. Biochem. J., **29**, 288–290.

Stickland, L. H. 1935 The oxidation of alanine by *Cl. sporogenes*. The reduction of glycine by *Cl. sporogenes*. Biochem. J., **29**, 889–898.

Woods, D. D., and Clifton, C. E. 1937 Hydrogen production and amino acid utilization by *Clostridium tetanomorphum*. Biochem. J., **31**, 1774–1788.

GROWTH FACTOR REQUIREMENTS OF BACILLUS LARVAE, WHITE[1]

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INTRODUCTION

For successful cultivation *in vitro* of *Bacillus larvae*, the organism responsible for American foulbrood of bees, media containing "natural" substances have heretofore been required, the organism failing to grow on ordinary laboratory substrates. White (1904) first cultivated this organism on a medium containing healthy bee larvae, though as prepared at first, using heat sterilization, the substrate permitted of but feeble growth. Using a broth of macerated larvae, filtered and added aseptically to a basal medium of nutrient agar, White (1907) obtained better growth, enabling him to conduct inoculating experiments leading to the establishment of *B. larvae* as the etiological agent. Later the same investigator (1919) prepared a medium to which egg yolk, removed aseptically from the shell, was added in place of the crushed brood. Maassen (1908) likewise succeeded in cultivating the organism, using crushed brood and also an agar substrate containing calf brain, egg albumin and peptone.

White's egg-yolk medium was modified by Sturtevant (1924) who found the addition of yeast helpful in promoting good growth. Testing the effect of plant extracts on growth of *B. larvae*, Lochhead (1928) found that satisfactory development could be obtained by replacing egg-yolk by extracts of orange, tomato or carrot, and developed a transparent medium containing carrot extract and yeast extract added to a base of peptone and K_2HPO_4. This medium has been used with success by Sturtevant (1932), Hitchcock (1936) and Stoilowa (1938). Later, Lochhead (1937) found an otherwise similar medium, in which turnip extract is used instead of carrot extract, to support rather better growth of *B. larvae* in the routine examination of suspected combs for this organism.

Tarr (1937) found a modification of the brood filtrate medium more satisfactory for obtaining large numbers of spores than other media on which *B. larvae* sporulates with difficulty. He (1938) also devised a chicken embryo medium which proved suitable for the germination of the spores of *B. larvae*. More recently Holst and Sturtevant (1940) have further modified carrot extract and yeast media, chiefly through the addition of cysteine.

Consideration of the media on which *Bacillus larvae* has been successfully cultivated suggests that accessory growth factors are required for the nutrition of the organism, since most of the addenda found necessary for growth contain substances, which, in the light of more recent nutritional studies, are required for various species of the more "fastidious" micro-organisms. The experiments

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here reported were planned to note more specifically the growth-factor requirements of the organism.

METHODS

For comparative purposes eight strains of *Bacillus larvae* were used, isolated from samples of American foulbrood comb ranging in age from fresh samples to those 13 years old, and including samples from widely separated parts of Canada. Cultures were isolated and tested for purity on turnip-extract-yeast-peptone agar. In the various tests the different strains behaved similarly, both in growth response and in morphological appearance, and hence are not reported individually.

The tests were made on slants of solid media for which washed agar was used (1.5 per cent). The various addenda were added to a basal medium similar to that used by West and Lochhead (1940) in nutritional studies of soil bacteria and which consisted of glucose, 1.0 gm.; K_2HPO_4, 1.0 gm.; KNO_3, 0.5 gm.; $MgSO_4$, 0.2 gm.; $CaCl_2$, 0.1 gm.; NaCl, 0.1 gm.; $FeCl_3$, 0.01 gm.; distilled water, 1000 ml. The solution was heated to boiling, filtered and the reaction adjusted to pH 6.8. Amino-acids were used at a concentration of 0.05 gm. per liter, peptone (Bacto-peptone) at 0.5 per cent concentration and growth factors as follows:

Thiamin	100	gamma per liter
Biotin	0.1	gamma per liter
Vitamin B_6	200	gamma per liter
Pantothenic acid	100	gamma per liter
Inositol	0 05	gm. per liter
Nicotinic acid	100	gamma per liter
Riboflavin	200	gamma per liter

Inoculation was made by spreading a standard loopful of suspension of *B. larvae* in uniform manner along the surface of the agar slant from top to bottom. Cultures were incubated at 37°C.

EXPERIMENTAL RESULTS

Amino acids, peptone and growth factors

Preliminary tests were made to note the adequacy of a mixture of amino-acids as compared with peptone, with and without the addition of growth factors, to support growth of *B. larvae*. For this purpose 10 amino-acids were used—cysteine, alanine, proline, asparagine, glutamic acid, aspartic acid, arginine, leucine, glycine and lysine. Growth factors consisted of a mixture of thiamin, biotin, vitamin B_6, pantothenic acid, inositol, nicotinic acid and riboflavin.

As noted in table 1, the combination of amino-acids, even with the addition of growth factors, was inadequate for growth. Peptone, though unsuitable by itself, supported very good development of *B. larvae* upon the addition of the combination of growth factors, with growth equal to that provided by such

"natural" media as carrot-extract-yeast-peptone agar. The slight growth observed with peptone alone consisted of atypical cells which were observed to undergo rapid disintegration preventing successful transfer from this medium.

Growth-factor requirements

With peptone added to the basal medium, a series of tests was made to note the effect of omitting individual growth factors from the combination. The results (table 2) were clear-cut in showing thiamin to be the essential factor. Without thiamin, growth was negligible, while the omission of each of the other growth factors from the combination was entirely without effect. Cysteine resulted in no growth response.

TABLE 1

Effect of amino-acids, peptone and growth factors

BASAL MEDIUM OF GLUCOSE-SALTS-NO_3 WITH ADDENDA AS BELOW	GROWTH OF B LARVAE (8 STRAINS)
Nil	–
10 amino-acids	–
7 growth factors	–
Amino-acids + growth factors	–
Peptone	Trace [1]
Peptone + growth factors	+++[2]

[1] Cells rapidly disintegrate, transfers soon sterile.

[2] Growth abundant, equivalent to that with addenda of vegetable extract, yeast or egg-yolk.

TABLE 2

Effect of omitting individual growth factors

BASAL MEDIUM OF GLUCOSE-SALTS-NO_3 WITH ADDENDA AS BELOW	GROWTH OF B LARVAE (8 STRAINS)
(1) peptone	Trace[1]
(2) peptone + cysteine	Trace
(3) peptone + cysteine + 7 growth factors	+++[2]
(4) (3), omitting thiamin	Trace
(5) (3), omitting biotin	+++
(6) (3), omitting vitamin B_6	+++
(7) (3), omitting pantothenic acid	+++
(8) (3), omitting inositol	+++
(9) (3), omitting nicotinic acid	+++
(10) (3), omitting riboflavin	+++

[1, 2] See footnotes to table 1.

To note any interrelationship between thiamin and the other growth factors, a further series of tests was made, in which the effect of thiamin alone and in combination with other growth factors was determined. As noted in table 3, thiamin alone is able to yield an excellent growth of *B. larvae*. Growth in the

presence of thiamin is not further improved by addition of any of the other factors individually or by the full combination of growth factors. The results indicate that in the presence of peptone thiamin by itself provides satisfactory growth conditions for the organism, and suggest that the "natural" substances employed for cultivating *B. larvae* depend for their effectiveness upon their content of thiamin.

TABLE 3

Effect of thiamin alone and with other growth factors

BASAL MEDIUM OF GLUCOSE-SALTS-NO_3 WITH ADDENDA AS BELOW	GROWTH OF B. LARVAE (8 STRAINS)
(1) peptone	Trace[1]
(2) peptone + 7 growth factors	+++[2]
(3) (2), omitting thiamin	Trace
(4) peptone + thiamin	+++
(5) peptone + thiamin + biotin	+++
(6) peptone + thiamin + vitamin B_6	+++
(7) peptone + thiamin + pantothenic acid	+++
(8) peptone + thiamin + nicotinic acid	+++
(9) peptone + thiamin + riboflavin	+++

[1, 2] See footnotes to table 1.

DISCUSSION

The suitability of peptone, as compared with the amino-acids used as basic ingredients, suggests the presence in it of other essential amino-acids, trace mineral elements or growth factors other than thiamin. Further experiments showed that in addition to the above-mentioned amino-acids, tryptophane, tyrosine, cystine, phenylalanine, histidine and serine were ineffective, even when peptone ash was incorporated in the media. Casein hydrolysate was unable to replace peptone.

While it has not been possible as yet to replace peptone by simpler sources of nitrogen and thus devise a medium of definitely known chemical composition, substitution of thiamin for the more complex "natural" addenda heretofore required makes possible the preparation of a comparatively simple medium for cultivating *B. larvae*. The inclusion of 0.01 per cent KNO_3 in the medium, an amount insufficient for a positive nitrite test with most nitrate-reducing species, though adequate for *B. larvae*, makes the medium suited to the characteristic nitrite reaction which has been found of considerable diagnostic value in detecting this organism (Lochhead 1928, 1937).

SUMMARY

Bacillus larvae, heretofore successfully cultivated only on media containing addenda of "natural" substances, has been found to grow well on a medium containing salts-sugar solution, peptone and thiamin.

Thiamin completely replaced the growth factor effect of such addenda as vegetable extract, yeast or egg yolk. Other growth factors, namely biotin,

vitamin B_6, pantothenic acid, inositol, nicotinic acid and riboflavin were without effect, nor did they increase the effect of thiamin in promoting growth either singly or in combination.

REFERENCES

Hitchcock, J. D. 1936 Laboratory and field tests of chlorine treatment of honey combs. J. Econ. Entomol., **29**, 895–904.

Holst, E. C., and Sturtevant, A. P. 1940 Relation of proteolytic enzymes to phase of life cycle of *Bacillus larvae*, and two new culture media for this organism. J. Bact., **40**, 723–731.

Lochhead, A. G. 1928 Cultural studies of *Bacillus larvac* White. Sci. Agr., **9**, 80–89.

Lochhead, A. G. 1937 The nitrate reduction test and its significance in the detection of *Bacillus larvae*. Can. J. Research, C, **15**, 79–86.

Maassen, A. 1908 Zur Aetiologie der sogenannten Faulbrut der Honigbienen. Arb. kaiserl. biol. Anstalt Land-. u. Forstw. **6** (Heft 1), 53–70.

Stoilowa, E. R. 1938 Vergleichende bakteriologische Untersuchungen an einigen deutschen Stämmen des *Bac. larvae*, des Erregers der bösartigen Faulbrut der Honigbiene. Zentr. Bakt. Parasitenk., II, **99**, 124–130.

Sturtevant, A. P. 1924 The development of American foulbrood in relation to the metabolism of its causative agent. J. Agr. Research, **28**, 129–168.

Sturtevant, A. P. 1932 Relation of commercial honey to the spread of American foulbrood. J. Agr. Research, **45**, 257–285.

Tarr, H. L. A. 1937 Studies on American foulbrood of bees. I. The relative pathogenicity of vegetative cells and endospores of *Bacillus larvae* for the brood of the bee. Ann. Applied Biol., **24**, 377–384.

Tarr, H. L. A. 1938 Studies on American foulbrood of bees. II. The germination of the endospores of *Bacillus larvae* in media containing embryonic tissues. Ann. Applied Biol., **25**, 633–643.

West, P. M., and Lochhead, A. G. 1940 The nutritional requirements of soil bacteria—a basis for determining the bacterial equilibrium of soils. Soil Sci , **50**, 409–420.

White, G. F. 1904 The further investigation of the diseases affecting the apiaries in the State of New York. N. Y. Dept. Agr. 11th Ann. Rept., p. 103–114.

White, G. F. 1907 The cause of American foulbrood. U. S. Dept. Agr. Bur. Entomol. Circ. 94.

White, G. F. 1919 Unheated egg-yolk media. Science, **49**, 362.

THE SANITARY SIGNIFICANCE OF PECTIN-FERMENTING, LACTOSE-FERMENTING, GRAM-NEGATIVE, NON-SPORE-FORMING BACTERIA IN WATER

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Although numerous workers have shown that gram-negative, non-spore-forming, lactose-fermenting, pectin-fermenting bacteria are widely distributed in nature, their sanitary significance remains uncertain. Bergey's Manual of Determinative Bacteriology (1939) describes the tribe Erwineae as plant parasites causing blights and soft rots, fermenting glucose and lactose with the formation of acid, or acid and a small amount of visible gas, and usually attacking pectin. Since these organisms resemble closely the coli-aerogenes group, those which produce visible gas might be expected to pass through the completed test in the bacteriological examination of water, and give false tests. Whether this group of plant pathogens can be differentiated from coli-aerogenes organisms of intestinal origin by their ability to ferment pectin cannot be stated until the occurrence of pectin-fermenting coli-aerogenes organisms in the intestinal tract of man and animals has been investigated. This study has been undertaken in order to determine: (1) a suitable medium for the testing of the ability of organisms to ferment pectin; (2) the incidence of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria in fecal material of man and animals; (3) the incidence of the above organisms in water; and (4) the cultural, morphological and biochemical characteristics of pectin-fermenting bacteria from the feces of man and animals, and from water.

Kruse (1910) referred to many investigators who had designated such common organisms as *Bacillus subtilis* and *Escherichia coli* as pectin-fermenting bacteria. Coles (1928) found that citrus pectin (heat sterilized) was fermented by seven out of thirteen strains of *Bacterium oxytocum*, four out of nine strains of *Bacterium aerogenes* and two strains of *Bacillus aceto-ethylicum*. He concluded that only organisms which commonly occur in the soil are capable of attacking pectin with the production of acid and gas. He also stated that none of the intestinal forms, classified in the sub-genus *Escherichia* of the genus *Bacterium*, produced acid or gas from pectin; however, he did find that some of the organisms belonging to the sub-genus *Aerobacter* were able to ferment pectin with the production of acid and gas. Burkey (1928) carried out an extensive study of pectin-fermenting organisms which had been isolated from soil, creek water, decayed potatoes, parsnips, cornstalks, hay infusions and sewage. He found that all of the gram-negative, lactose-fermenting organisms which possessed the ability to ferment citrus pectin (heat sterilized) produced both acid and gas from it. All of these organisms had characteristics similar to those of coliform organisms, including the ability to form typical colonies on Endo and eosin-methylene-blue agars.

He classified all of them in the genus *Aerobacter* but drew no conclusions as to their sanitary significance.

Werch, Day, Jung, and Ivy (1941), in a study of the role of the intestinal bacteria in the decomposition of pectin, used a broth containing a one-per-cent solution of pure citrus pectin which had been sterilized by filtration. Although their work dealing with the isolation of the responsible bacteria, with the determination of the exogenous or endogenous source of the pectinase and with the products of decomposition was not completed, they concluded that pectin is decomposed by bacteria present in the feces.

The ability of the various species of *Erwinia* to ferment pectin is too well known to require reviewing.

EXPERIMENTAL

1. Development of suitable media for testing the ability of organisms to ferment pectin

Chemically pure apple pectin obtained from the General Foods Sales Company, New York, was used in this investigation. This pectin was shown to be free from reducing substances other than pectin by two methods. In the first method a 70 per cent ethyl alcohol extract was tested with Benedict's solution; in the second method the pectin was precipitated from a 2 per cent solution and the filtrate was deleaded with hydrogen sulfide and tested with Benedict's solution. Controls containing small amounts of arabinose and galactose gave positive qualitative Benedict tests by both methods.

Since no definite information could be found concerning the effect on pectin of sterilization by heat, the effect of autoclaving a one per cent solution was investigated. At pH 5.0, pH 6.0 and pH 6.5, the pectin was hydrolized to reducing substances. At pH 7.0 only traces of reducing substances were produced, but at pH 7.2 and pH 7.5 large amounts of reducing substances were again produced.

Since from these experiments it was concluded that it would be unsafe to test the ability of organisms to ferment pectin using a pectin-containing medium which had been sterilized by heat, the sterilization of the pectin was carried out in the following manner. Five grams of pectin were added, under aseptic conditions, to a sterile one-liter Erlenmeyer flask. To this were added 20 ml. of 75 per cent ethyl alcohol, under aseptic conditions, and the flask was rotated rapidly to mix the contents. Since a high concentration of alcohol was undesirable in the final medium it was necessary to allow the alcohol to evaporate from the sterilized pectin before proceeding to the next step. It was found that, if the flask was allowed to remain at room temperature for 5–8 days, the alcohol would evaporate from the flask and leave the pectin with just a thin coating of alcohol. This coating of the grains of pectin with alcohol proved to be desirable for, when 500 ml. of sterile distilled water were added to the flask, the pectin dispersed into the water at once, giving a one-per-cent solution which was clear and free of clumps. This one-per-cent solution could be added to any basal medium aseptically and used for the determination of the ability of organisms to ferment pectin. In this investigation a basal medium containing 4 grams of ammonium

chloride and 4 grams of dibasic potassium phosphate per liter of distilled water, and one containing 6 grams of Bacto beef extract and 10 grams of peptone per liter of distilled water, were used. It was necessary to prepare these basal media in the above double strengths and to so adjust the pH of each that, after the addition of an equal quantity of the alcohol-sterilized pectin solution, the desired concentration of the ingredients would be obtained and the final pH of each medium would be 7.1. A combination indicator containing brom-cresol-purple and cresol red was used. Two milliliter amounts of the basal medium were placed in Durham fermentation tubes and sterilized, after which 2 ml. amounts of sterilized pectin solution were added aseptically. After the addition of the pectin, the tubes of media were placed in the incubator at 37° C. for 48 hours and then at room temperature for 4 days, in order to detect contamination.

Since, throughout these studies, neither acid nor gas production was ever observed in the extract pectin broth, except for acid production in check *Erwinia* cultures, the results with this medium will be omitted from the tabulations. As acid was frequently produced in duplicate inoculations in the synthetic medium, it would appear that fermentation was probably masked in the extract broth.

2. The incidence of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria in human and animal feces

Eosin methylene-blue agar streak plates were made from feces samples from 56 humans, 7 cows, 3 mules and 7 dogs and from the caecal contents of 6 fowls. After a 48 hr. incubation period well isolated colonies were picked from the plates into duplicate tubes of both extract pectin broth and synthetic pectin medium. Two or more colonies of each type present were picked from each plate. Transfers were made from all colony types which, if they had appeared on a plate from water, would have been considered to be typical or atypical colonies of coliform organisms. All cultures so isolated will be referred to as strains, even though many of the cultures were apparently duplicates. Controls consisted of uninoculated tubes and of duplicate tubes inoculated with a known pectin-fermenting *Erwinia* and with a known non-pectin-fermenting fecal strain of *Escherichia coli*. The number of samples studied, their source, the number yielding pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria, the number of strains tested for ability to ferment pectin, and the number which fermented synthetic pectin medium with the production of acid are included in table 1.

None of 507 strains of organisms which were isolated from 56 human stools fermented pectin. Gram-negative, lactose-fermenting, non-spore-forming organisms with the ability to form acid in the synthetic pectin medium were isolated from the feces of one of seven cows, one of three mules, and from four of seven dogs. None were isolated from the samples from three horses and six fowls. In all, of 477 strains which were isolated from animal feces, only 48 produced acid in the synthetic pectin medium. None produced gas. Thirty-eight of the 48 strains were from dogs. It is possibly significant that the dogs were laboratory animals which had been fed only on prepared chow. The majority of the 48 strains of pectin fermenting organisms were from colonies which

resembled those of *Aerobacter*; none were from colonies which resembled those of typical *Escherichia*. However, not all of the organisms which produced aerobacter-like colonies were able to ferment pectin.

All of the three strains which had been isolated from cow feces proved to be methyl-red positive. Two gave the "Imvic" reactions ++−+ and appeared to be *Escherichia* intermediates. The other, when first isolated, produced gas and acid from lactose. However, when its ability to ferment lactose was tested for the second time, using carefully sterilized lactose broth, it failed to produce gas. This may have been due either to the loss of the ability to produce gas, or to the lactose broth used in the first test having been injured by the process of sterilization. This strain gave the "Imvic" reactions −+−−, and appeared to be an *Escherichia* organism. Four strains which had been isolated from mule feces proved to be methyl-red negative and Voges-Proskauer positive. The "Imvic" reactions −−++ were obtained with three of the strains, and +−++ with one. All of these appear to be *Aerobacter* strains. The characteristics of three strains which, on preliminary examination, appeared to be identical with some of the strains which were studied, were not investigated in detail. All of the 38 strains which were isolated from dog feces gave the "Imvic" reactions −−++, and appeared to be typical strains of *Aerobacter aerogenes*. All of the strains which were motile showed a peritrichous arrangement of flagella.

TABLE 1

The incidence of gram-negative, pectin-fermenting, lactose-fermenting, non-spore-forming bacteria in human and animal feces

SOURCE OF SAMPLES	NO. OF SAMPLES EXAMINED	NO OF SAMPLES YIELDING PECTIN FERMENTERS	NO. OF STRAINS PICKED FROM E M B PLATES AND TESTED FOR ABILITY TO FERMENT PECTIN	NO. OF STRAINS WHICH FERMENTED PECTIN (SYNTHETIC MEDIUM)
Humans	56	0	507	0
Cows	7	1	92	3
Horses	3	0	68	0
Mules	3	1	44	7
Dogs	7	4	155	38
Fowls	6	0	118	0
Totals	82	6	984	48

The abilities of the 48 pectin-fermenting strains to ferment lactose, sucrose, glucose, maltose, alpha-methyl-glucoside, cellobiose, levulose, mannose, galactose, raffinose, rhamnose, trehalose, melezitose, salicin, amygdalin, aesculin, xylose, arabinose, glycerol, mannitol, dulcitol, sorbitol, adonitol, inositol, erythritol, glycogen, dextrin, inulin and soluble starch were determined. Their fermentation characteristics proved to be of little value in their identification.[1]

[1] For results of fermentation tests see The Sanitary Significance of Pectin-fermenting, Lactose-fermenting, Gram-negative, Non-spore-forming Organisms.—D. B. McFadden, Thesis, University of Kentucky.

3. The incidence of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria in water

For the attempted isolation of pectin-fermenting coliform organisms from water, samples from a number of different sources were collected according to the Standard Methods of Water Analysis, 1936, and taken to the laboratory where they were examined as quickly as possible. As this study of pectin-fermenting coliform organisms in water was made with the view not only to determine the incidence of these organisms in water, but also to determine if colonies of these organisms would appear on eosin methylene-blue plates following the usual procedure of enriching water samples in lactose broth, the following method for the isolation of these organisms was selected.

Duplicate tubes containing 10 ml. amounts of Standard Methods double-strength lactose broth were inoculated with 10 ml. portions of the water sample, and duplicate tubes of single-strength Standard Methods lactose broth were inoculated with 1.0 ml. and 0.1 ml. portions of the sample. The contents of the tubes which showed gas production at the end of 48 hours were streaked on Standard Methods eosin methylene-blue agar and, after incubation for 48 hours, well isolated colonies were picked from the plates to nutrient agar slants. At least two colonies of each type present on the plates were picked. Each culture thus obtained was considered to be a strain. Since the incidence of pectin-fermenting organisms in water was not known, enrichment with pectin broth was also used as an aid in the isolation of these organisms from the first samples which were examined. Ten milliliters of the sample were placed in duplicate tubes of double-strength extract pectin broth and double-strength synthetic pectin medium. The concentration of pectin in these media was one per cent and each tube contained 10 ml. of the medium. One milliliter and one tenth milliliter portions of the sample were placed in duplicate tubes of single-strength extract pectin broth and synthetic pectin medium. The contents of tubes which showed the production of acid, or acid and gas after 7 days incubation were streaked out on eosin methylene-blue agar plates, and well isolated colonies were picked from these plates after 48 hours' incubation. All of the strains which were isolated were tested for their ability to ferment pectin and lactose.

Eleven samples of water were taken from ponds, wells, a cistern, a lily pool, a creek and a boiler compound, and examined for the presence of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria. The results are presented in table 2.

From table 2 it can be seen that all of the samples except three yielded gram-negative, lactose-fermenting, pectin-fermenting, non-spore-forming bacteria. Of 259 strains which were isolated from the eosin methylene-blue plates, 71 produced acid from pectin and acid and gas from lactose. Thirty-five of these strains were isolated following pectin enrichment and 36 following lactose enrichment. All of the 71 strains would have been isolated by the Standard Methods of Water Analysis procedure and would have been considered to belong to the coli-aerogenes group.

Detailed characteristics of 51 of the 71 gram-negative, lactose-fermenting, pectin-fermenting, non-spore-forming organisms which were isolated from water have been studied. Nineteen, which were obviously duplicates of some of those that have been studied, were given only preliminary study. The 51 strains fall into eleven groups according to their "Imvic" reactions. In the first group are four strains, with the reactions $++--$, which appear to be typical strains of *Escherichia coli*. The second group consists of six strains, with the reactions $-+--$, which appear to be indole-negative *Escherichia*. The next three groups appear to be *Escherichia* intermediates. In the third group are ten strains with the reactions $++-+$, in the fourth four strains with the reactions $-+++$ and in the fifth two with the reactions $++++$. Of thirteen strains

TABLE 2

Incidence of gram-negative, pectin-fermenting, lactose-fermenting, non-spore-forming bacteria in water

SOURCE OF SAMPLE	NO. OF COLONY TYPES ON E M.B. AGAR	NO OF COLONIES PICKED FROM E M.B. PLATES	NO. OF STRAINS FERMENTING PECTIN (SYNTHET MED)	NO. OF STRAINS FERMENTING PECTIN AND LACTOSE	NO OF STRAINS FROM PECTIN ENRICHMENT, FERMENTING PECTIN AND LACTOSE	NO OF STRAINS FROM LACTOSE ENRICHMENT FERMENTING PECTIN AND LACTOSE
Pond	8	22	17	17	13	4
Lily pool	3	19	5	0	0	0
Pond	8	33	2	1	0	1
Pond	10	36	14	10	9	1
Creek	28	45	21	16	13	3
Boiler compound	7	20	1	0		0
Well	3	10	2	0		0
Well	5	16	10	2		2
Cistern	3	20	13	13		13
Well	8	22	6	4		4
Source unknown	3	16	8	8		8
Totals		259	99	71	35	36

in the sixth group, with the reactions $--++$, eleven have the characteristics of *Aerobacter aerogenes* and two the characteristics of *Aerobacter cloacae*. In go up seven are six strains, with the reactions $+-++$, which might be considered to be indole-producing strains of *Aerobacter*. The one strain in group eight gives the reactions $--+-$. Although the fermentation characteristics[2] of the strains in the remaining groups are typical of the coli-aerogenes group, the "Imvic" reactions make their relationships uncertain. In group nine are three strains with the reactions $---+$, in group ten one strain with the reactions $+--+$ and in group eleven one strain with the reactions $----$.

[2] For results of fermentation tests see The Sanitary Significance of Pectin-fermenting Lactose-fermenting, Gram-negative, Non-spore-forming Organisms.—D. B. McFadden, Thesis, University of Kentucky.

DISCUSSION

Burkey (1928) and Coles (1928) reported that some strains of coliform organisms were able to produce both acid and gas from a synthetic pectin medium. On the other hand none of the strains which we have encountered produce gas from synthetic pectin medium. Since we have shown that heat sterilization produces reducing substances from pectin and since both of these investigators employed heat-sterilized media, it is possible that the gas was produced from some product which had resulted from hydrolysis of the pectin.

Extract pectin broth appears to be unsatisfactory for the detection of fermentation by coliform organisms. Apparently fermentation is masked by the production of alkali in the medium. When the pH values of extract pectin broth cultures were determined electrometrically, they were found to have increased, whereas the pH values of the synthetic pectin medium cultures had dropped to as low as pH 4.82 after seven days incubation. The strain of *Erwinia* which was used as a control produced acid in both media.

The strains of pectin-fermenting coliform organisms which we have isolated from animal feces and from water appear to be somewhat more varied than those which were obtained from water by Burkey. He classified all of his strains in the genus *Aerobacter*. Of the strains which we have isolated from animal feces, those from the cow were methyl-red-positive intermediates while those from mules and dogs were typical strains of *Aerobacter aerogenes*. The strains which we have obtained from water include a number which appear to be intermediates, three strains of typical *Escherichia coli* and many strains of *Aerobacter*.

We have classified all of our strains of pectin-fermenting coliform organisms in the genera *Escherichia* and *Aerobacter* although it is possible that some of the strains may belong in the genera *Erwinia* and *Serratia*. According to Bergey's Manual of Determinative Bacteriology (1939) it would appear that organisms in the genus *Erwinia* differ from those in the tribe Eschericheae by usually fermenting pectin and by being pathogenic for plants. The results of our study of pectin-fermenting organisms seem to point out that the ability to ferment pectin is possessed by a great many members of the coliform group and that, therefore, this characteristic is not of much value in identifying members of the genus *Erwinia*. No complete record of the characteristics of the members of the genus *Erwinia* is available. Without knowledge of the reactions of these organisms on the media which are commonly used for the study of organisms in the Eschericheae group it does not appear to be possible to determine if any of the coliform organisms which are obtained from water are *Erwinia*.

Pederson and Breed (1928) in a study of the fermentation of glucose by organisms of the genus *Serratia*, came to the conclusion that non-pigmented strains of *Serratia* might be properly classed in the genus *Aerobacter*. Accordingly, some of the pectin-fermenting strains from water, which rapidly liquefy gelatin and utilize uric acid, may be non-pigmented strains of *Serratia*.

At present, it is not possible to state what is the sanitary significance of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming organisms in

water. However a high percentage of the organisms which we have isolated from water, using Standard Methods procedure, ferment pectin, whereas none from human feces and only a few from animal feces ferment pectin. It appears likely, therefore, that many of the pectin-fermenting organisms which we have isolated from water were not of fecal origin. According to the present Standard Methods of Water Analysis a water supply would be condemned on the basis of the presence of these organisms.

SUMMARY

The incidence of pectin-fermenting, lactose-fermenting, gram-negative, non-spore-forming bacteria in the feces of man and of animals and in water has been studied. Pectin-fermenting ability has been tested in extract broth and synthetic bases to which alcohol-sterilized pectin had been added aseptically. Pectin-fermenting strains have been detected by the production of acid in a synthetic medium. No visible fermentation occurred in the extract broth medium.

Of 507 strains of coliform organisms which were isolated on eosin methylene-blue agar from the feces of 56 persons, 68 strains which were isolated from the feces of three horses and 118 strains which were isolated from the feces of 6 fowls, none fermented pectin. Of 92 strains which were isolated from the feces of seven cows, three, all of which were isolated from the same cow, fermented pectin. Of 44 strains which were isolated from the feces of 3 mules, 7, all of which were isolated from the same mule, fermented pectin. Of 115 strains which were isolated from the feces of 7 dogs, 38, which were isolated from 4 of the 7 dogs, fermented pectin.

In order to determine the incidence of pectin-fermenting coliform organisms in water, pectin fermentation tests have been made on strains which were obtained from colonies on eosin methylene-blue agar plates that had been made following the enrichment of water samples in Standard Methods lactose broth and in pectin enrichment broth. Of 259 strains which have been isolated, 71 have been found to ferment pectin. Of these, 35 were from plates which had been streaked from pectin enrichment media and 36 from plates which had been streaked from Standard Methods lactose broth.

Four of the pectin-fermenting strains, all of them isolated from water, possessed typical *Escherichia coli* characteristics, 28 strains, from mule and dog feces and from water, possessed typical *Aerobacter aerogenes* characteristics and 32 strains, from cow feces and from water, possessed characteristics of intermediate coliform organisms.

Due to lack of information concerning the reactions of members of the genus *Erwinia* and of non-pigmented strains of *Serratia* on the media which are used for studying coliform organisms, it has been impossible to determine if any of the pectin-fermenting organisms which have been isolated belong to either of these genera.

It has been concluded that since relatively more pectin-fermenting coliform organisms are found in water than in the feces of animals, some of them, at least, are probably not of fecal origin.

REFERENCES

Bergey, D. H. 1939 Manual of Determinative Bacteriology. 5th Ed. Baltimore, Williams & Wilkins.

Burkey, L. A. 1928 The fermentation of cornstalks and their constituents. I. Studies on the pectin-fermenting bacteria. Iowa State Coll. J. Sci., **3**, 57–100.

Coles, H. W. 1928 The digestion of pectin and methylated glucoses by various organisms. Plant Physiol., **1**, 379–384.

Kruse, W. 1910 Allgemeine Mikrobiologie. Leipzig. Cited from Burkey, L. A 1928, Iowa State Coll. J. Sci., **3**, 57–100.

Pederson, Carl S., and Breed, Robert S. 1928 The fermentation of glucose by organisms of the genus Serratia. J. Bact., **16**, 163–183.

Werch, S. C., Day, A. A., Jung, R. W. and Ivy, A. C. 1941 Rôle of the intestinal bacteria in the decomposition of pectin. Proc. Soc. Exptl. Biol. Med., **46**, 569–573.

THE EFFECT OF STAPHYLOCOCCUS ENTEROTOXIN ON ISOLATED RABBIT GUT SEGMENTS

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It is the purpose of this paper to report some observations on the response of rabbit intestinal muscle to the enterotoxin extracted from strains of staphylococcus cultured from food known to have produced attacks of food poisoning. The cultural, epidemiological and other aspects of this subject have been discussed by several authors (Barber, 1914; Dack, Cary, Woolpert and Wiggers, 1930; Jordan, 1931; Jordan and Burrows, 1934) and the literature has been reviewed by Richmond (1939).

Except for descriptions of the clinical symptoms, indicating general gastrointestinal irritation, little attention has been given to the physiological mechanisms involved in this train of sequelae. The rapid onset suggested muscular disturbance, consequently the first approach was made in experiments on segments of rabbit intestine.

More recently Bayliss (1940) has published an experimental study of enterotoxin emesis in kittens. A brief paragraph in this paper refers to experiments similar to ours on cat and rabbit intestinal strips, from which the conclusion was drawn that the enterotoxin has no direct effect on smooth muscle. No detailed data were given. However, in the description of the gross responses of the animals to injections of enterotoxin, Bayliss mentions defecation and loss of appetite. The postmortem examinations showed "excessive mucus" in the tract. These observations certainly suggest local irritation. Bayliss finally concluded that the main action of enterotoxin is on peripheral sensory structures, secondly on the vomiting center, with no important influence on the musculature. In view of the paucity of data on intestinal strips we do not feel that our observations are controverted. Since we did no experiments on intact animals we have no data on emesis. The two studies, therefore, deal mainly with different mechanisms, both of which are involved in the response to enterotoxin by the intact organism.

EXPERIMENTAL TECHNICQUE

Contiguous segments of jejunum, about 4 cm. long, were isolated immediately after the rabbit was killed by a blow on the neck, and suspended in oxygenated Ringer solution at pH 7.3 and 38°C. To determine the rôle of the

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mucosa, one segment was closed by ligatures and the everted rosettes of mucosa trimmed off completely. A fine hypodermic needle was used to relieve internal air pressure when necessary. The other segment was suspended so that the fluid circulated freely through the lumen.

A constant volume of 60 ml. of solution was maintained in the bath for each segment. Before the test solution was added, an equivalent volume of Ringer solution was siphoned off.

The enterotoxin was prepared by the technique of Dack and Woolpert (1933). Cultures were grown on semisolid veal infusion medium in an atmosphere of high CO_2 content, the toxin separated by Berkefeld filter, and adjusted to pH 7.3 in a constant volume of Ringer solution, and enclosed in serum bottles until required.

Two types of control material were used. One consisted of extracts made by the same technique from cultures of air-borne strains of staphylococcus. The other was prepared in an identical manner from uninoculated medium.

TABLE 1

TOXIN	INCREASED ACTIVITY		NO CHANGE IN ACTIVITY		DECREASED ACTIVITY		TOTAL NO TRIALS
	Number	Per cent	Number	Per cent	Number	Per cent	
Mucosa exposed	39.0	72.3	14.0	26.0	1.0	1 8	54
Mucosa not exposed	42 0	77.8	11.0	20 3	1.0	1 8	54

EXPERIMENTAL RESULTS

In 54 trials with the enterotoxin applied to the strip with the mucosa not exposed to the toxin, 77.8 per cent of the trials showed an increase in the activity of the gut segment, 20.3 per cent showed no change, and 1.8 per cent showed a decrease, while in the same number of trials with the mucosa exposed to the toxin, 72.2 per cent of the trials showed an increase in activity, 26.0 per cent showed no change, and 1.8 per cent showed a decrease (table 1). The criteria adopted as a measure of increased activity were primarily increased tonicity, as measured by an elevation of the base line, and increased amplitude. The rate of the contractions was not altered significantly. In almost all instances increased activity meant an increase in tonicity (fig. 1), in a very few an increase in amplitude only, and in many an increase in both tonicity and amplitude. Some strips showed persistently increased tonus after addition of the toxin, while others showed evidence of rhythmic spasms as a characteristic response.

Control material was added to the intestinal strips in the same concentration and under the same conditions. Generally, the control material was applied to strips of gut which had also been subjected to the enterotoxin. Of 40 trials with the control broth extract on the strips with the mucosa not exposed, 55.0 per cent showed no alteration of activity, 27.5 per cent showed a decreased activity (fig. 2), and 17.5 per cent showed increased activity, while in a similar number of trials on the strips with the mucosa exposed, 60.0 per cent showed

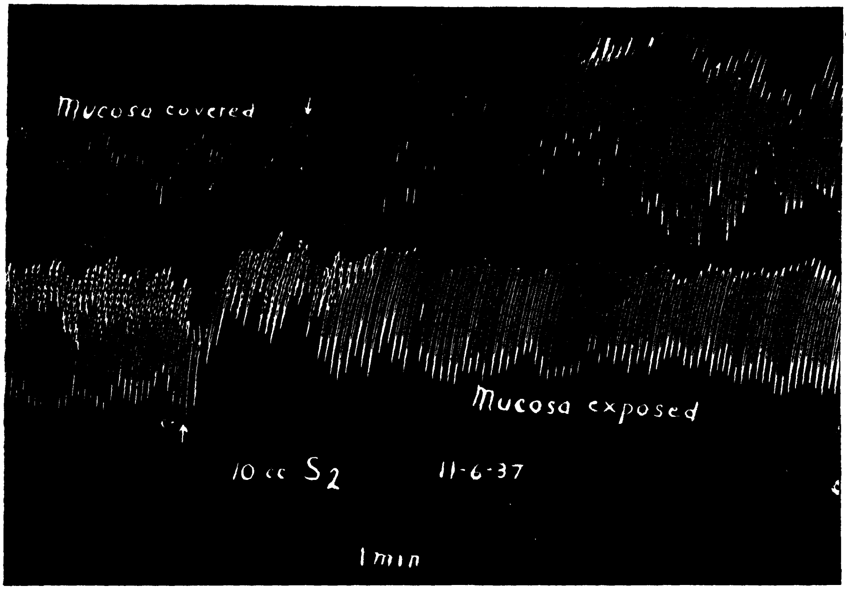

FIG 1 UPPER TRACING, GUT SEGMENT CLOSED, LOWER, MUCOSA EXPOSED
The response is similar in both. At arrows 10 ml of enterotoxin in broth filtrate S_2 was added. The culture was isolated from a strain of milk-borne Staphylococcus

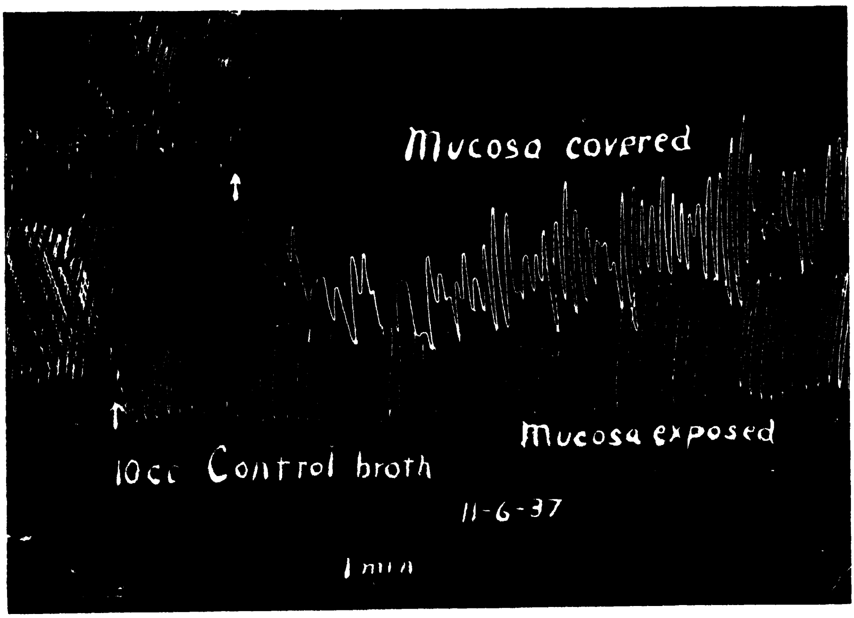

FIG 2. SAME SEGMENTS AS IN FIGURE 1
Ten milliliter of control were added to each. The response in each is comparable, characterized by decreased tonus and amplitude

no change in activity, 22.5 per cent showed a decrease, and 17.5 per cent showed an increase (table 2). A preparation obtained from a strain of *Staphylococcus* isolated from the air, as far as could be determined, acted in the same manner as the control broth extract. From our data, the observation of Dolman and his collaborators that the toxin need not be absorbed through the mucosa of the intestine to produce its effect appears to be borne out, since the responses of the strips with the mucosa exposed were practically the same, both quantitatively and qualitatively. The slight quantitative differences (never exceeding 6 per cent) in the number of responses of the two strips of gut subjected to the same conditions are entirely within the range of experimental error. Hence, we feel that the effect of the toxin on the smooth muscle of the gut is probably that of a non-specific irritant.

In addition to these results there were 102 trials with single open segments, of extracts cultured from 18 samples of food known to have caused poisoning. Of these, 80, or 78.4 per cent, responded with definitely increased motor activity. The remainder either showed no change or so little as to be inconclusive. A contiguous segment of gut was used as a control indicator for uninoculated broth extract. There was a mild augmentation of activity in only 15 trials.

TABLE 2

CONTROL	INCREASED ACTIVITY		NO CHANGE IN ACTIVITY		DECREASED ACTIVITY		TOTAL NO. TRIALS
	Number	Per cent	Number	Per cent	Number	Per cent	
Mucosa exposed	7.0	17.5	24.0	60.0	9.0	22.5	40
Mucosa not exposed	7.0	17.5	22.0	55.0	11.0	27.5	40

DISCUSSION

That many investigators have concerned themselves with the development of cultural characteristics specific for enterotoxin-producing strains of staphylococci with little success, is apparent from review of the literature. Therefore, it is readily understood that for the present, it would be decidedly advantageous to have some other method for the identification of food-poisoning strains. Ultimately it may be determined that no uniformity of cultural characteristics for these strains exists and that they have only one common feature—the ability to produce enterotoxin. Dolman *et al.* (1936) have already developed an index for the presence of the enterotoxin by observing symptoms of food poisoning in kittens injected intraperitoneally with the enterotoxin. This method is dependent upon individual differences in the animals and upon subjective interpretations of what constitute food poisoning symptoms in kittens. Therefore, it is suggested that the possibility of the utilization of a technique for recording the increased tonicity of the smooth muscle of the rabbit gut by the enterotoxin as observed in these experiments, in the absence of any extraneous smooth muscle irritant, be investigated further as a more objective method of demonstrating the presence of the enterotoxin.

Although one cannot interpret clinical facts directly in the light of *in vitro* experiments, these experiments tend to explain many of the features of the clinical syndrome of *Staphylococcus* food poisoning. Thus, the nausea and epigastric distress seen early in the clinical syndrome perhaps reflect the early tonic effects of the enterotoxin upon the small intestine. Further, if the marked increase in the tonicity of the smooth muscle in response to the enterotoxin *in vitro* be considered analogous to clinical enterospasm, much of the severe abdominal pain experienced at the height of an attack can be accounted for, since it is well known that enterospasm is an important factor in the production of gastro-intestinal pain. Although the mechanical factor of increased activity of the gastro-intestinal tract undoubtedly plays a significant rôle in the production of the diarrhea observed in the syndrome, the physico-chemical effects of the enterotoxin must undoubtedly play the major rôle in this response as well as in the genesis of the type of shock observed in severe cases. Further pharmacological experimentation will be necessary to explain these more generalized effects of the enterotoxin.

CONCLUSIONS

1. Experiments indicate that the *Staphylococcus* enterotoxin produces predominantly an increase in tonicity of the smooth muscle of the rabbit gut *in vitro*.
2. The increased tonicity of the smooth muscle of the rabbit gut produced by the *Staphylococcus* enterotoxin may be comparable to clinical enterospasm, to which much of the gastro-intestinal pain experienced in food poisoning may be attributed.
3. The observation of Dolman and his collaborators (1936) that the *Staphylococcus* enterotoxin need not be absorbed through the intestinal mucosa to produce its effects on the smooth muscle of the gastro-intestinal tract appears to be confirmed by our experiments. Also, the effect of the enterotoxin on the smooth muscle of the gut is probably that of a non-specific irritant.
4. The possibility of the utilization of the technique of recording the effect of the enterotoxin on smooth muscle as an index of the enterotoxin producing ability of various strains of staphylococci should be investigated further.

REFERENCES

BARBER, M A. 1914 Milk poisoning due to a type of *Staphylococcus albus* occurring in the udder of a healthy cow. Philippine J. Sci., **9**, 515–519.

BAYLISS, M. 1940 Studies on the mechanism of vomiting produced by staphylococcus enterotoxin. J. Exptl. Med., **72**, 669–684.

DACK, G. M., CARY, W. E., WOOLPERT, O. C., AND WIGGERS, H. 1930 An outbreak of food poisoning proved to be due to a yellow hemolytic staphylococcus. J. Preventive Med., **4**, 167–179.

DOLMAN, C. E., WILSON, R. J., AND COCKCROFT, W. H. 1936 A new method of detecting staphylococcus enterotoxin. Can. Pub. Health J., **27**, 489–493.

JORDAN, E. O. 1931 Staphylococcus food poisoning. J. Am. Med. Assoc., **97**, 1704–1707.

RICHMOND, J. B. 1939 The effect of the staphylococcus enterotoxin on the motility of the isolated smooth muscle of the rabbit gut. Thesis, University of Illinois, pp. 1–21.

WOOLPERT, O. C., AND DACK, G. M. 1933 Relation of gastro-intestinal poison to other toxic substances produced by staphylococci. J. Infectious Diseases, **52**, 6–19.

TECHNIQUE FOR THE DETERMINATION OF THE SENSITIVITY OF A STRAIN OF STREPTOCOCCUS TO BACTERIOPHAGE OF TYPE A, B, C OR D

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In a previous publication (1934) four serologic types[1] of streptococcal bacteriophage were described and designated A, B, C and D, to conform with the nomenclature of phage specific to other genera of bacteria.

It has been shown (Evans) that phage in the nascent state, i.e., in the presence of cells of a sensitive strain undergoing lysis, is more potent than in the filtered state. The nascent and also the filtered phage may be utilized for the differentiation of certain streptococcic groups for which one or another of the several types of phage have special affinities. Phage A is an exception, however, in that it has not been found useful in classification studies because in the nascent state it is capable of lysing more or less completely all strains of Lancefield's Group A; the filtered phage A_{751} may or may not attack the strains of the various subgroups of Group A, irrespective of other characters.

Phage B is specific in its reactions and may be utilized to identify the streptococci of Lancefield's Group C, which are the only ones sensitive to filtered phage B. Although the strains of Groups A and E are resistant to filtered phage B, they are sensitive to it in the nascent state, with few exceptions to that general rule. Streptococci of the other Lancefield groups are completely resistant to Phage B (Evans and Verder, 1938).

Phage C has a special affinity for the strains of *Streptococcus scarlatinae* (unpublished data) with which it gives clear lysis in high dilution of filtered phage. Filtered phage C lyses many of the strains of other streptococcic species of group A, but with notably less vigor than it lyses the strains of *S. scarlatinae*. The strains of the epidemicus group are characterized by resistance to phage C (Evans, 1940).

Filtered phage D has a special affinity for the enterococci, effecting complete lysis in a wide range of dilutions, whereas only an occasional strain of Group A is slightly sensitive to filtered phage D. Many strains of group A are slightly sensitive to phage D in the nascent state, but correlation of this with other characters of group A has not been observed (unpublished data).

TECHNIQUE

In preparing the stock supply of phage the streptococcic sub-stratum is always the same for a given type of phage, and is specified in the designation of phage.

[1] In this series of publications "race" refers to a pure phage of any isolation, being analogous to the use of the term "strain" in bacteriologic literature; "type" refers to the serologic identity of the race as determined by neutralization by specific serum.

The phages used in our studies are designated A_{751}, B_{563}, C_{594} and D_{693}, the figure referring to the strain of streptococcus which served for the propagation of the phage.

The technique for determining the sensitivity of a given strain of streptococcus to phage as previously described (Evans, 1936) has been modified and is therefore described here again in detail. This modified technique may be used to determine the sensitivity of any strain of streptococcus to phage A, B, C or D.

The test for sensitivity of a strain of streptococcus to one of the types of phage can be most clearly described by an example. The testing of strain 1501 for sensitivity to phage C_{594} requires 11 tubes, each containing 4.5 ml. of neopeptone broth. To each of 9 of the tubes of broth (3 sets of 3 tubes each) is added 0.5 ml. of filtered phage in such dilution that the final dilutions are 10^{-1}, 10^{-3} and 10^{-5}.

Overnight culture is used for the inoculum. It is diluted by transferring a loopful to a tube containing 4.5 ml. of broth. The inoculum is one drop of diluted culture. An example of a protocol is given in table 1 which shows the

TABLE 1

Examples of a test for sensitivity of a strain of streptococcus to phage C_{594}

STREPTOCOCCIC INOCULUM	APPEARANCE OF BROTH	DILUTED PHAGE FILTRATE		
		10^{-1}	10^{-3}	10^{-5}
Strain 594	Turbid	4	4	4
Strain 1501	Turbid	0	0	0
Strains 594 and 1501	Turbid	0	2	3

0 designates turbidity as in the control broth culture.
1, 2, and 3 designate decreasing degrees of turbidity.
4 designates complete lysis.

data for the test to determine the sensitivity of Strain 1501 to phage C_{594}. The tubes with diluted phage of one set and a tube of broth are inoculated with Strain 594. These tubes act as a control test to determine the activity of the sample of phage and the viability and sensitivity of Strain 594. Clearing should occur in all three tubes containing phage. The tubes of diluted phage of another set and a tube of broth are inoculated with Strain 1501. The tubes with diluted phage of the third set are inoculated with both strains, 1501 and 594. The cultures are incubated about 18 hours, and then readings are made for lysis. The method of making the readings is illustrated in table 1 which shows that Strain 594 (of the species of *S. scarlatinae*) is highly sensitive to phage C; Strain 1501 is resistant to the filtered phage, but it is lysed by phage C in the nascent state, under certain conditions such as those which prevailed in the second and third tubes of the series.

The enhanced activity of nascent phage was the subject of a previous publication (Evans, 1940). It is illustrated by the sensitivity of Strain 1501 to phage C_{594} at the moment of its development, although it is resistant to the filtered phage.

The purpose of testing with three dilutions of phage, varying from low to high, is to provide a wide range of conditions for the unknown vagaries of sensitivity of the strain in question, because zoning of the reaction as illustrated in table 1 is common. In the case of any race of streptococcic phage, lysis by the filtered or nascent phage may occur in the higher when it is not evident in the lower dilution, or, lysis may occur in the lower but not in the higher dilutions containing a weaker concentration of phage.

SUMMARY

The technique for the determination of sensitivity of a strain of streptococcus to bacteriophage is described. The same technique is used for the determination of the sensitivity of any strain of streptococcus to any of the types of streptococcic phage A, B, C or D.

REFERENCES

EVANS, ALICE C. 1934 Streptococcus bacteriophage: a study of four serological types. U. S. Pub. Health Repts., **49**, 1386–1401.

EVANS, ALICE C. 1936 Studies on hemolytic streptococci I. Methods of classification. J. Bact., **31**, 423–437.

EVANS, ALICE C. 1940 The potency of nascent streptococcus bacteriophage B. J. Bact., **39**, 597–604.

EVANS, ALICE C. 1940b Studies on hemolytic streptococci. VI. The epidemicus group. J. Bact., **40**, 215–222.

EVANS, ALICE C., AND VERDER, ELIZABETH 1938 Studies on hemolytic streptococci. V. The characteristics of human and animal strains of groups A and C. J. Bact., **36**, 133–147.

ANOTHER SEROLOGIC TYPE OF STREPTOCOCCIC BACTERIOPHAGE

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In a study of a group of streptococci of Lancefield's Group A, biochemical characters, including fermentative reactions and sensitivity to bacteriophage, were investigated. It was observed that the strains of two of the sub-groups: (1) the lactose-deficient group (Evans, 1941), and (2) the mannitol-fermenting group were not particularly sensitive to any of the 4 known types of streptococcic phage. It appeared likely that one or more new types of phage might be found if a search were made, using strains of streptococci of these two groups for the substrata.

EXPERIMENTAL

The methods used for the isolation of phage from sewage, and the techniques for the study of its characters, followed, with certain modifications, those previously described (Evans, 1934).

Isolation. Samples of sewage collected for another purpose in February and March, 1940, from the final clean-out trap leading from hospitals to the city sewage system were studied. Samples from Baltimore, Boston, New York City, Evansville, Indiana, and Washington, D. C. were examined for phage which might be active against any of six selected strains of streptococci representing the two mentioned sub-groups.

The sewage was treated in such a manner as to propagate any phage particles with an affinity for the selected strains which might be present. It was used as one of the ingredients of a medium in which the streptococci were grown in serial cultures.

The medium consisted of meat-infusion broth of double strength distributed in test tubes in 8 ml. amounts and sterilized by heat. Previous to the inoculation, 8 ml. of sewage sterilized by filtration was added to each tube and 4 ml. of a sterile filtrate was also added. The filtrate will be described presently.

The inoculum for the several culture generations of streptococci in the sewage medium was in every instance a mixture of the six strains. The mixture was prepared by adding one drop of overnight broth culture of each strain to 9 ml. of broth. One drop of this diluted mixed culture was planted in the sewage medium. After growth overnight the culture in sewage medium was filtered, and this filtrate was tested to demonstrate any possible lytic activity by planting each of the six strains in a tube of broth containing 10 per cent of the filtrate.

The 4 ml. of filtrate added to the sewage medium for the first of the series of mixed streptococcus cultures was from a broth culture inoculated with the unfiltered sewage and incubated overnight at 37°C. For the second and follow-

ing culture generations of streptococcus the 4 ml. of filtrate added to the medium was from the preceding culture of the series. The process was continued until six or more serial plantings had been made. The filtrate from each of the serial cultures was tested with each of the six strains of streptococcus for evidence of lysis.

A lytic agent active against the mannitol-fermenting strain No. 985 was obtained from two of the sewage samples; after the first enrichment passage in the medium containing Baltimore sewage, and after the third enrichment passage in medium containing the Evansville sewage. No lytic agent was found capable of attacking the other five strains included in the experiment.

Strain 985 was received from Dr. Griffith in 1935, labelled "Beatty, type 17." It was found to be serologically distinct from all other type strains of Griffith, thus agreeing with the results reported by Griffith, and also by Keogh, Simmons and Wilson.

After several serial passages of the new races of phage in culture with strain 985, the filtrates of each race contained the lytic agent in dilutions as high as 10^{-6}.

The new races of phage were tested for neutralization by antiserum specific to phages of the serologic types A, B, C or D, using a technique previously described (Evans, 1934). Neither of the new races was neutralized by any one of the antiserums. This showed that the new races did not belong to any of the known serologic types.

An antiserum was then prepared by injecting a rabbit with several doses of the filtered Evansville phage according to the technique previously described. The antiserum from this rabbit neutralized the new Baltimore race of phage as well as the Evansville race, but it neutralized none of the phages of types A, B, C or D. These results showed that the two new races belonged to the same type, which differed serologically from types A, B, C and D. The new serologic type was designated E.

DESCRIPTION OF PHAGE E

Phage E differs from the four previously known serologic types of streptococcus phages A, B, C and D in its unusual resistance to heat, and in the rapidity of development of secondary cultures.

Thermolability. The temperature of inactivation of a freshly prepared sample of phage E_{985} was determined in triplicate tests. The titer was 10^{-6}; the pH was 7.4. One-half-ml. quantities were sealed in 5 mm. tubes and immersed for one hour in a water-bath at a constant temperature. Each cooled sample was then added to 4.5 ml. of neopeptone broth which was inoculated with one drop of homologous culture At the same time a control tube containing broth was inoculated with the culture. The tubes were incubated and read frequently for lysis. If no lysis occurred the culture was filtered and the filtrate was tested for active phage. This process was continued through 3 serial passages in broth culture, after which it was concluded that inactivation was complete if no evidence of lysis was observed.

Phage E_{985} survived temperatures up to 74–75°C. but after heating at 75–76°C.

no lysis occurred. The temperature of inactivation was thus shown to be higher by 10 degrees for phage E than for any other known streptococcic phage. It had previously been shown that phages A_{751} and B_{563} were inactivated at 60°C.; phage C_{594} was inactivated at 65°C. and phage D was inactivated at 63°C. A test to determine the thermolability of one of the previously described phages, made simultaneously with a test on phage E_{985}, confirmed the earlier observation.

Secondary cultures. The development of secondary growth in cultures lysed by phage E_{985} was rapid, appearing in 2 to 3 hours after complete lysis. On account of the rapidity of the development of secondary growth it was necessary to modify the technique described for determining sensitivity to phage A, B, C or D (Evans, 1942). Instead of planting the tubes containing phage E with a drop of diluted culture they were planted with a drop of undiluted overnight culture. Incubation was for 6 hours, after which the tubes were placed in a cold room at about 15°C. and readings were made the following day.

The plaques formed by phage E on 1.25 per cent agar containing 0.5 per cent glucose, with streptococcus 985 as the substratum, ranged between 0.5 and 0.75

TABLE 1

Correlation between the characters of mannitol fermentation and sensitivity to phage E_{985}

MANNITOL FERMENTATION	SENSITIVITY TO FILTERED PHAGE E_{985}			
	+		−	
	Number	Per cent	Number	Per cent
+	21	52.5	19	47 5
−	33	12 9	222	87 1

mm. in diameter. The edges were blurred and irregular. As in broth cultures, secondary growth developed rapidly.

Range of activity. The strains of Lancefield's Group A were generally lysed more or less completely by nascent phage E_{985} with only an occasional resistant strain found. Hence, sensitivity to nascent phage E_{985} was found to be a character of no value for the differentiation of streptococci. Filtered phage E_{985} lysed the homologous strain in dilutions as high as 10^{-6}. Of the 295 heterologous strains of Lancefield's Group A tested for sensitivity to filtered phage E_{985}, 54 were found to be more or less sensitive. Of the 295 strains 40 fermented mannitol and 255 did not. That there is a correlation between mannitol fermentation and sensitivity to phage E_{985} is indicated in table 1 which shows that the frequency of sensitivity to phage E_{985} in mannitol-positive strains was more than four times that in mannitol-negative strains.

Of the 54 strains which showed more or less sensitivity to phage E_{985}, none but the homologous strain was lysed completely in all 3 tubes of the test. The unique high degree of sensitivity of strain 985 to phage E is in agreement with its unique agglutinogenic property. Of the 295 strains tested for sensitivity to phage E_{985}, 163 were also tested for agglutinability by an antiserum prepared by treating a rabbit with a course of injections with strain 985. Only the

homologous strain was agglutinated by the serum after absorption to remove non-specific agglutinins according to Griffith's technique. Since it has been found that in streptococci of other serologic types sensitivity to phage is correlated with serologic grouping it is to be expected that when another streptococcus of type 17 is encountered it will be found to be sensitive to phage E_{985}.

SUMMARY

Two races of streptococcic bacteriophage, serologically alike and distinct from the 4 previously known serologic types A, B, C and D, were recovered from sewage.

The new type is designated E. Among 295 strains of streptococci tested, only one was found to be highly sensitive to the new race of phage. This strain, No. 985 of Griffith's type 17, is serologically distinct from 163 strains of streptococcus which were tested for agglutinability in antiserum 985.

REFERENCES

Evans, Alice C. 1934 The prevalence of streptococcus bacteriophage. Science, **80**, 40–41.

Evans, Alice C. 1934 Streptococcus bacteriophage: A study of four serological types. U. S. Pub. Health Repts., **49**, 1386–1401.

Evans, Alice C. 1941 Cross protection between heterologous agglutinogenic types of beta hemolytic streptococci. J. Immunol., **42**, 15–34.

Evans, Alice C. 1942 Technique for the determination of the sensitivity of a strain of streptococcus to bacteriophage of races A, B, C, or D. J. Bact., **44**, 207–209.

Griffith, F. 1935 The serological classification of *Streptococcus pyogenes*. J. Hyg., **34**, 542–584.

Keogh, E. V., Simmons, R. T., and Wilson, Harold. 1941 *Streptococcus pyogenes* (Group A). A review of 1,341 strains collected from various human sources. Australian J. Exptl. Biol. Med. Sci., **19**, 51–55.

RESPIRATORY STUDIES OF THE MICROCOCCI[1]

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Respiratory enzymes play an important part in many of the fundamental reactions of the cell and since there has been only a very limited amount of work done upon the respiratory enzymes of the micrococci, the present study was undertaken.

LITERATURE SURVEY OF THE DEHYDROGENASES

Braun and Vasarhelyi (1932) found that relatively few substrates were dehydrogenated by *Staphylococcus aureus.*

Fabre (1935) studied the dehydrogenases produced by *S. aureus.* Of the 67 substrates tested only 24 were found to be active. The most actively dehydrogenated were glucose, mannose, galactose, sucrose, lactose, xylose, maltose, lactate and formate. Substrates weakly activated were succinate, fumarate, ethyl alcohol and glutamate. The hydrogen donors which were inactive or doubtful were dulcitol, rhamnose, acetate, citrate, oxalate, alanine, phenylalanine, asparagine, and aspartate.

Ehrismann (1937) made a study of the dehydrogenases of micrococci and streptococci. The dehydrogenase activity was determined for *S. aureus* and *Staphylococcus albus*, *Streptococcus pyogenes*, *Micrococcus tetragenus* and *Micrococcus candicans.*

Most of the substrates were found to be activated by all the organisms, the difference being quantitative rather than qualitative. The main exceptions were the inability of *M. candicans* to activate malate, ethyl alcohol and arabinose; of *M. tetragenus* to activate dulcitol; and of *S. aureus* to dehydrogenate asparagine.

EXPERIMENTAL

Study of dehydrogenase activity of the micrococci

The dehydrogenase activity of the various strains of micrococci was determined by the Thunberg (1918) technique.

The organisms were grown in Roux bottles at 20°C. for 30 to 34 hours on a medium containing 0.5 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent peptonized milk, 0.2 per cent meat extract, and 3.0 per cent agar, pH 7.0–7.1. The growth was removed and washed three times by centrifugation with saline and suspended in saline in a bottle fitted with a tube for sterile aeration. The cell suspension was stored at five degrees and aerated before use.

[1] Journal Article No. 549 N.S.

[2] Summary of thesis presented in partial fulfillment of the Ph.D. degree by the senior author.

In the Thunberg tube were placed 1 ml. substrate (adjusted to pH 7.05), 1 ml. saline, 1 ml. of 1–5000 methylene blue, and 1 ml. of phosphate buffer, and in the hollow stopper 1 ml. of aerated cell suspension. The buffer used was a mixture of M/30 K_2HPO_4 and M/30 KH_2PO_4 with a pH of 6.85.

The tubes were evacuated for 2.5 minutes with constant shaking and incubated in water at 40°C. After allowing for equilibrium the cells were tipped into the tube and the time required to bring about complete reduction of the methylene blue was noted. The cell suspension was diluted with saline so that the reduction time, in the absence of any added substrate, was about one hour. The figures given in all the tables represent an average of two determinations. In no case did the difference between the two exceed five per cent and in most cases it was much less.

The relative rates of dehydrogenation are shown in table 1, where glucose is the standard, 100. Raffinose, maltose, sucrose, glucose and fructose were readily activated. *Micrococcus flavus* was more active against carbohydrates than any other organisms since all the sugars except d-arabinose and d-xylose were dehydrogenated at a rate superior to that of glucose. *Micrococcus aurantiacus* dehydrogenated lactose and galactose at a greater rate than did the other organisms. *Micrococcus freundenreichii* actively oxidized only glucose, and *Micrococcus cinnebareus* only glucose, raffinose, and fructose.

Ethyl alcohol and d-mannitol were readily activated by all the organisms except *M. freudenreichii* which did not activate any of the alcohols to an appreciable extent. Dulcitol would be classed as "fair to good" as a substrate for dehydrogenation by *M. flavus* and *M. cinnebareus*, but only "poor" in the case of the other organisms; while glycerol would be classed as "good to excellent" with *M. flavus* and *M. aurantiacus*, but "poor to inert" in the case of the other organisms.

Of the four-carbon-dicarboxylic acids, citrate, lactate, acetate, and malate were activated at an appreciable rate, while fumarate, tartrate, and formate served as poor donators to methylene blue. Oxalate was not activated by *M. luteus* and *M. flavus* and served as a very poor substrate for the other organisms. With *M. aurantiacus* and *M. cinnebareus* formate was found to serve as an excellent hydrogen donor, while *M. cinnebareus* activated only formate, tartrate, acetate, and lactate to an appreciable extent.

The amino acids used were not activated to any great degree with the exception of glutamate and α-alanine which, together with asparagine, served as "good to excellent" substrates. Beta alanine was not activated as rapidly as was alpha alanine but was superior to the majority of amino acids.

Study of the complete respiratory system of the micrococci

The various strains of micrococci were tested by the Warburg apparatus to determine the rate of oxygen uptake in the presence of the different substrates. The organisms were cultivated as for the dehydrogenase studies and were stored at 5°C. and aerated each time before use.

Before determining the oxygen uptake with the different substrates the opti-

mum conditions for respiration were determined. It was found that the oxygen uptake was practically independent of the concentration of phosphate buffer

TABLE 1

The relative rates of dehydrogenation, with glucose taken as the standard, equal to 100

SUBSTRATE	M. LUTEUS	E. FLAVUS	M. AURANTIACUS	M. FREUNDENREICHII	M CINNEBAREUS
d-Glucose	100	100	100	100	100
d-Arabinose	58	78	85	13	6
l-Arabinose	49	103	74	17	12
d-Xylose	51	90	78	41	17
d-Fructose	132	121	78	77	75
d-Mannose	84	126	73	59	43
d-Galactose	49	110	174	73	13
Lactose	68	101	124	53	3
Maltose	400	195	132	65	43
Sucrose	147	399	80	76	43
Raffinose	334	147	250	85	77
l-Rhamnose	57	117	65	81	18
Ethyl alcohol	140	523	3743	24	105
Glycerol	56	114	367	24	32
Dulcitol	64	138	60	18	95
d-Mannitol	106	119	134	18	154
Formate	50	81	1462	13	99
Acetate	137	124	162	24	69
Lactate	144	138	638	21	60
Citrate	160	166	93	33	6
Oxalate	40	69	69	9	5
Malate	130	146	88	9	10
Succinate	177	180	155	73	9
Fumarate	121	128	83	9	4
Maleate	208	172	199	11	40
Tartrate	117	84	60	9	82
Asparagine	179	138	205	31	13
a-Alanine	196	126	153	31	27
b-Alanine	113	121	90	10	9
Glycine	105	86	82	15	6
dl-Leucine*	73	94	75	26	7
dl-Isoleucine*	63	72	82	34	3
dl-b-Phenylalanine*	54	85	65	15	59
d-Glutamate*	125	90	228	57	41
Aspartate*	52	70	117	24	46
Endogenous respiration	40	69	51	8	4

* Substrate used in M/30 concentration.

(K_2HPO_4–KH_2PO_4) between M/10 and M/90 concentrations. Temperature and pH exerted a greater effect. The optimum pH was found to be between 6.3 and 6.8, the optimum temperature between 35° and 39°C. At 37°C. and above, the

rate of oxygen uptake decreased with time, so respiration studies were conducted at 35°C. which showed about the same initial rate as the higher temperatures yet decreased the tendency for heat inactivation. Using glucose as a representative substrate it was found that the respiratory system of the cells was quite stable since the oxygen uptake remained constant for four days if the cells were stored and aerated at low temperatures.

In the light of the above facts, respiration studies were conducted for 60 minutes at 35°C. in the presence of M/30 phosphate buffer (mixture of M/30 K_2HPO_4 and M/30 KH_2PO_4, pH 6.7). The substrates were made up M/30 and adjusted to pH 7.05. The washed cell suspension was stored at 5°C. and aerated for 30 minutes at 5°C. before use.

In the Warburg vessel were placed 1 ml. of aerated cells, 1 ml. buffer pH 6.7, 0.6 ml. saline, 1 ml. substrate M/30, and in the center cup 0.2 ml. of 20 per cent NaOH to absorb the carbon dioxide evolved. Mixtures of buffer, saline and substrate showed no oxygen uptake in the absence of cells.

Nitrogen determinations were made on each cell suspension by the Kjeldahl-Gunning method. The indicator used was a mixture of methylene blue and methyl red as suggested by Johnson and Green (1930). The oxygen uptake was corrected to standard temperature and pressure and was reported as Q_{O2} (N) which represents the cubic mm. oxygen uptake per hour per milligram of nitrogen.

Sucrose, maltose, and glucose were excellent hydrogen donors (table 2) when oxygen was used as the ultimate hydrogen acceptor. *M. flavus* was more active against the sugars than were the other organisms. *M. flavus* alone was very active against rhamnose and *M. cinnebareus* was very active against fructose.

Ethyl alcohol was rapidly oxidized by all the organisms as was glycerol except in the case of *M. luteus*. Dulcitol was activated at an appreciable rate by *M. flavus*, while mannitol served as an excellent substrate for *M. cinnebareus*, although both were poor substrates for the other organisms.

Succinate and lactate were generally oxidized at a very rapid rate while fumarate, acetate, and malate were readily activated only by *M. luteus*, *M. flavus*, and *M. cinnebareus*. *M. aurantiacus* and *M. freundenreichii* oxidized lactate readily but were not very active against the other acids.

Glutamate and asparagine were oxidized rapidly by all the organisms. Alpha alanine was activated at a rate greater than that of beta alanine by all the micrococci except *M. aurantiacus* which oxidized both at the same rate.

The oxygen uptake of most of the substrates was constant or decreased slightly with time, although with a few of the substrates the initial rate of respiration increased during the 60 minute test. This is shown in figure 1. Glutamate showed a slightly increasing oxidation rate with all the organisms except *M. flavus*. Acetate and dl-b-phenylalanine showed an increasing rate with *M. flavus*, *M. cinnebareus*, and *M. freundenreichii* as did glycine and aspartate in the presence of *M. aurantiacus*. The initial lag noticed in the case of these substrates suggests the formation of some intermediate compound which provides a better substrate for respiration.

Study of respiratory inhibitors

Several respiratory inhibitors have been used in an attempt to reveal certain properties and modes of action of enzymes. Narcotics have been found to

TABLE 2

The rate of oxygen uptake by micrococci

The figures represent the $Q_{O_2}(N)$ or the Mm³ oxygen/hour/mgm. nitrogen.

SUBSTRATE	M. LUTEUS	M. FLAVUS	M. AURANTIACUS	M. CINNEBAREUS	M. FREUDENREICHII
d-Arabinose	36.5	27.7	31.3	63.0	56.5
l-Arabinose	39.1	40.4	30.3	64.1	55.9
d-Xylose	56.5	130.3	32.5	74 3	65.4
d-Glucose	145.7	79.5	141.7	318.7	204.0
d-Fructose	67.9	40.7	99.4	465.8	74 1
d-Mannose	62.9	40.3	51.9	108.4	134.6
d-Galactose	98.7	138.3	35.8	70.2	60.8
Lactose	81.3	139.2	7.3	54.6	57.4
Maltose	145.8	193.4	41.1	371.5	180.0
Sucrose	201.8	143.2	390.4	421.2	164.1
Raffinose	58.9	163.0	35.6	79 4	66.5
l-Rhamnose	53.3	147.5	25.1	51.2	46.8
Ethyl alcohol	150.0	194 8	333.1	406.7	552.5
Glycerol	46.3	151.4	279.6	378.5	230.1
Dulcitol	95.0	112.3	18.7	45.5	46.7
d-Mannitol	62.6	43.5	71.3	415.7	37 6
Formate	43.0	55.0	127.8	207 9	34 1
Acetate	141.6	170.7	57.0	511.7	32.3
Lactate	63.5	60.1	242.4	537.6	333.8
Citrate	43.9	137.9	19.0	41.6	51.9
Oxalate	79.5	122.1	18.5	39 8	49.9
Malate	131.8	127.8	22.9	343 7	57.8
Succinate	189.7	429.0	51 9	408.5	66.9
Fumarate	174.2	313.0	33.1	292.5	63.7
Maleate	43.4	34.5	19.8	58.2	36.9
Tartrate	67.6	106.6	24.4	28.1	41 7
Asparagine	137.0	126.0	77.2	477.1	151.5
a-Alanine	102.8	161.7	33 5	338.9	465 6
b-Alanine	93.9	118.8	33.9	94 7	49.2
Glycine	116.3	134.3	154.6	237.8	60.0
dl-Leucine	56.9	37.3	32.6	57.5	41.4
dl-Isoleucine	79.4	82.0	39.6	129.5	174.5
dl-b-Phenylalanine	179.7	177.8	24.0	439.6	80.0
d-Glutamate	141.2	306.1	288.4	613.5	477.6
Aspartate	47.2	162.8	61 9	168.9	84 1
Endogenous respiration	41.8	23.1	16.2	38.1	31.5

inhibit the dehydrogenases and are used to identify such systems. Cyanide has been found to inhibit iron-catalyzed reactions, hence reacts with cytochrome-

oxidase, catalase and peroxidase, while it is considered to have little effect upon the dehydrogenases, according to Green and Brosteaux (1936), and Hawthorne and Harrison (1939). It has been found, however, that the l-malic dehydrogenase of *Escherichia coli* is sensitive to higher concentrations of cyanide (Gale and Stephenson, 1939); as are many dehydrogenation reactions performed with intact cells (Green and Brosteaux, 1936; Ehrismann, 1937).

There is little information concerning the action of sodium azide on bacterial dehydrogenases although it inhibits catalase, peroxidase and cytochrome-

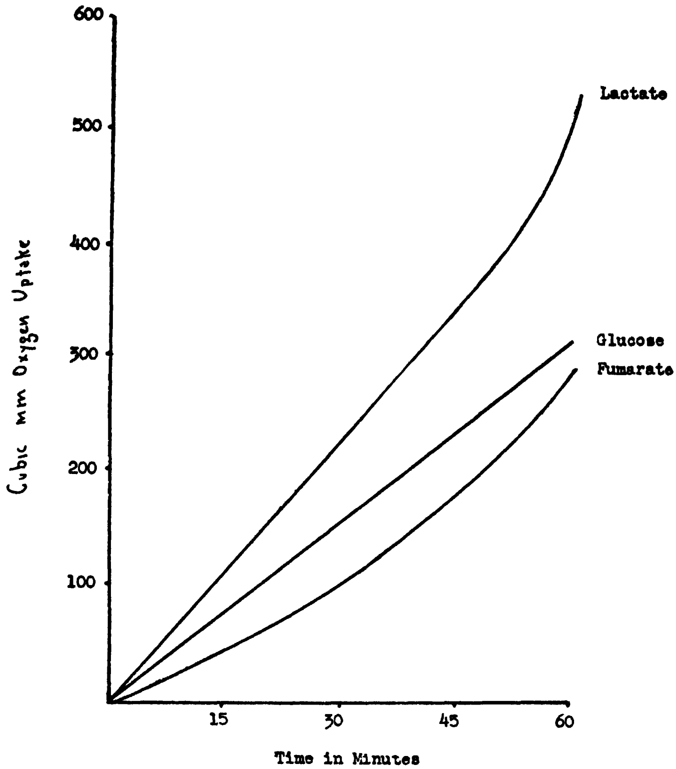

FIG. 1. THE RATE OF OXYGEN UPTAKE BY M. CINNEBAREUS

oxidase. The mono-halogen derivatives of acetic acid have been reported as inhibiting the reactions occurring during fermentation, Das (1937).

Ehrismann (1937) studying the effect of cyanide on the dehydrogenase activity of *Staphylococcus aureus* found that M/100 cyanide decreased the reduction time of methylene blue (increased the dehydrogenase activity) in the presence of lactate, glucose and fructose but inhibited the oxidation of glycine and alanine. Lactate and amino acid dehydrogenation were inhibited about 50 per cent by M/10 cyanide. Lower concentrations exerted correspondingly less action on lactate while a concentration as low as M/1000 still inhibited the dehydrogenation of the amino acids.

The effect of different concentrations of sodium monobromacetate on *Staphylococcus aureus* was determined in the presence of the above substrates. The activation of glucose and fructose was inhibited more than 50 per cent by M/100 sodium monobromacetate, while the oxidation of glycine and alanine was only slightly inhibited. However, in the presence of M/10 sodium bromacetate the dehydrogenation of lactate was stimulated while the activation of glucose and fructose was completely inhibited.

Influence of inhibitors upon dehydrogenases

Method. The inhibitors sodium azide, sodium cyanide and sodium monochloracetate were studied by placing in the Thunberg tube 1 ml. of cells, 1 ml. M/30 buffer, pH 7.03, 0.3 ml. saline, 0.7 ml. of the inhibitor (M/10 to M/10,000), 1 ml. methylene blue 1–20,000, and in the stopper 1 ml. of M/10 substrate.

When cyanide was used as the inhibitor, the tubes were set up as above and the substrate added after 20 minutes. The time was noted for complete reduction of the methylene blue. When sodium azide and sodium monobromacetate were used as inhibitors, the cells, methylene blue, saline and inhibitor were allowed to stand ten minutes before the addition of the substrate. The controls showed that none of inhibitors used reduced the methylene blue in the presence of buffer, saline, and substrate.

A great difference was found among the organisms in regard to their dehydrogenase sensitivity toward inhibitors (table 3). The dehydrogenases of *M. luteus* and *M. flavus* were stimulated by a cyanide concentration as high as 1.4×10^{-2} molar while the other organisms were generally inhibited by this concentration. *M. aurantiacus* showed the greatest sensitivity toward cyanide, being inhibited by a concentration of 1.4×10^{-4} molar.

M. cinnebareus and *M. flavus* were the least sensitive to sodium monochloracetate since they were generally stimulated by a concentration of 1.4×10^{-2} molar, while *M. aurantiacus* appeared to be most sensitive to this concentration.

Sodium azide appeared to be a stronger inhibitor than sodium cyanide since a concentration of 1.4×10^{-3} molar inhibited the dehydrogenases to a greater extent than did this concentration of cyanide.

The glucose dehydrogenase of *M. aurantiacus* appeared to be exceptionally resistant to both sodium monochloracetate and sodium azide while the other dehydrogenases of this organism appeared to be very sensitive to all inhibitors. The amino acid dehydrogenases were found to be uniformly more sensitive to all inhibitors than were the dehydrogenases active against the sugars and fatty acids.

Influence of inhibitors upon oxygen uptake by micrococci

Callow (1924) in studying the oxygen intake of a number of bacteria found very little oxygen uptake for *Staphylococcus aureus* and *Sarcina aurantiaca*. Gerard (1931) found the Q_{O_2} (c.mm. oxygen uptake per hour per mgm. dry weight of cells) of *Sarcina lutea* to be between four and six, while the Q_{O_2} in glucose was about 20. Methylene blue increased the oxygen uptake of endogenous respira-

TABLE 3

The per cent increase or decrease in dehydrogenase activity in the presence of inhibitors

INITAL CONCENTRATION	M/10	M/100	M/1000	M/10,000
FINAL CONCENTRATION	1.4×10^{-2}	1.4×10^{-3}	1.4×10^{-4}	1.4×10^{-5}
Potassium cyanide				
M. luteus				
Glucose	+64	+71		
Sucrose	+104	+29		
Lactate	+48	+115	+47	
Succinate	+14	+18		
Glycine				−37
dl-b-Phenylalanine				−52
M. flavus				
Glucose	+225	+105		
Sucrose	+246	+217		
Lactate	+133	+156	+184	
Succinate	+129	+107		
Glycine				−65
dl-b-Phenylalanine				−80
M. aurantiacus				
Glucose	−31	−36		
Sucrose	−56	−48	−37	
Lactate	−57		−5	
Succinate	−60	−57		
Glycine			−80	
dl-b-Phenylalanine				−67
M. cinnebareus				
Glucose	−22	+52		
Sucrose	−36	+44		
Lactate	−34		+116	
Succinate	−15		+4	
Glycine				−50
dl-b-Phenylalanine				−5
M. freundenreichii				
Glucose	−25	+324		
Sucrose	−14	−6		
Lactate	+2.4	±0		
Succinate	+157	+500		
Glycine				−74
dl-b-Phenylalanine				−68
Sodium monochloracetate				
M. luteus				
Glucose	−31	−30		
Sucrose	−28	−22		
Lactate	−10	−16		
Succinate	−3	+13		
Glycine				−31
dl-b-Phenylalanine				−58

TABLE 3—(*Continued*)

INITIAL CONCENTRATION	M/10	M/100	M/1000	M/10,000
FINAL CONCENTRATION	1.4×10^{-2}	1.4×10^{-3}	1.4×10^{-4}	1.4×10^{-5}
Sodium monochloroacetate				
M. flavus				
Glucose	+18	+7.1		
Sucrose	−7.2	−11		
Lactate	−8.8	+6.4		
Succinate	+25	+28		
Glycine				−59
dl-b-Phenylalanine				−66
M. aurantiacus				
Glucose	+59			
Sucrose	−46	+23		
Lactate	−26	−5		
Succinate	−56	−6.9		
Glycine				−81
dl-b-Phenylalanine				−66
M. cinnebareus				
Glucose	−35	−24		
Sucrose	+24	+33		
Lactate	+11			
Succinate	−11	+51		
Glycine				−50
dl-b-Phenylalanine				−5
M. freundenreichii				
Glucose	−18	−5.4		
Sucrose	−18	−7.8		
Lactate	−24	−38		
Succinate	−31			
Glycine				−39
dl-b-Phenylalanine				−80
Sodium azide				
M. luteus				
Glucose		−12	−4	
Sucrose		−29		
Lactate		+6	−20	
Succinate		−40	−16	
Glycine				−27
dl-b-Phenylalanine				−62
M. flavus				
Glucose		+22		
Sucrose		+15	+36	
Lactate		+17	+16	
Succinate		−3 3		
Glycine				−47
dl-b-Phenylalanine				−71

TABLE 3—(*Continued*)

INITIAL CONCENTRATION	M/10	M/100	M/1000	M/10,000
FINAL CONCENTRATION	1.4×10^{-2}	1.4×10^{-3}	1.4×10^{-4}	1.4×10^{-5}
Sodium azide				
M. aurantiacus				
Glucose		+64		
Sucrose		−32	+3.5	
Lactate		−29		
Succinate		−40	−6.4	
Glycine			−16	
dl-b-Phenylalanine				−77
M. cinnebareus				
Glucose		−39	−8	
Sucrose		+26		
Lactate		+160		
Succinate		−32	+50	
Glycine				−61
dl-b-Phenylalanine				−40
M. freudenreichii				
Glucose		+3.7		
Sucrose			−55	
Lactate			−38	
Succinate			−60	
Glycine				−60
dl-b-Phenylalanine				−60

tion about 50 per cent while the oxidation of lactate was inhibited between 10 and 30 per cent. It was found that cyanide in M/100 concentration did not inhibit the oxygen uptake of the cells in the presence of any of the substrates. A cyanide concentration of M/10 decreased respiration about 65 per cent. Working with the same strain one year later Barron (Gerard 1931), however, found that M/100 cyanide inhibited respiration about 50 per cent.

Cook, Haldane and Mapson (1931) found that concentrations of cyanide below 2×10^{-3} molar did not bring about serious inactivation of the dehydrogenases of *Escherichia coli.* With formate as the substrate it was found that cyanide strongly inhibited oxygen uptake, but that the addition of methylene blue together with cyanide resulted in an oxygen uptake nearly that of the formate alone in the absence of the inhibitor. With lactate as the substrate, methyllene blue alone (M/250) inhibited respiration about 74 per cent, and when cyanide and methylene blue were added together about 67 per cent of the original uptake (lactate alone) was obtained.

After studying the effect of the various inhibitors upon the dehydrogenases, the effect of the inhibitors upon the complete respiratory mechanism of the cell was determined.

Method. In the Warburg vessel were placed 1 ml. cells, 1 ml. buffer pH 7.05, 0.1 ml. saline, and 0.5 ml. inhibitor which was adjusted to pH 7.1 and in the center cup 0.2 ml. of 20 per cent NaOH to take up the carbon dioxide evolved.

When sodium monochloracetate and sodium azide were used, the cells, buffer, saline, and inhibitor were allowed to stand, with frequent agitation, for ten minutes before adding the substrate; when cyanide was used the substrate was added after 20 minutes. The carbon dioxide absorbent used was the KOH-KCN mixture suggested by Krebs (1935).

A mixture of buffer, saline, substrate, methylene blue and inhibitor showed no oxygen uptake under the conditions of the experiment.

Using glucose as a representative substrate it was found that *M. luteus* was rather resistant to sodium cyanide. Figure 2 indicates that a cyanide concentration of 1.4×10^{-2} molar inhibited respiration nearly 70 per cent. A concentration of 1.4×10^{-3} molar inhibited respiration slightly during the first 45 minutes, but the total oxygen uptake at the end of 60 minutes was identical with the uptake in the absence of inhibitor. *M. aurantiacus* likewise showed a similar increase when glucose was used as the substrate, but no other organisms or substrates acted in such manner.

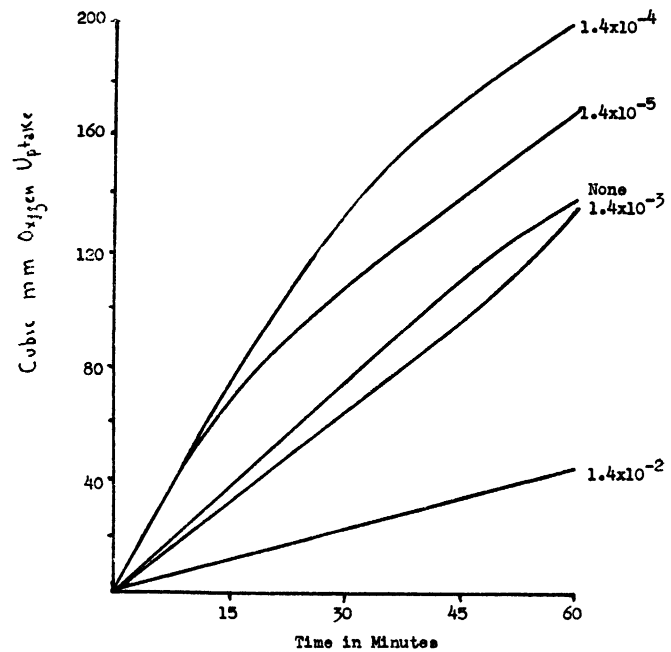

FIG. 2. THE EFFECT OF POTASSIUM CYANIDE (IN MOLAR CONCENTRATION) UPON THE OXYGEN UPTAKE OF M. LUTEUS

Cyanide concentrations less than 1.4×10^{-3} molar brought about a stimulation. This amounted to 43 per cent in a cyanide concentration of 1.4×10^{-4} molar while half this amount of cyanide stimulated only 21 per cent.

The influence of inhibitors on the oxygen uptake of the organisms was determined using as substrates glucose, sucrose, lactate, succinate, glycine, and dl-b-phenylalanine. A concentration of inhibitor was chosen which did not

exert too pronounced an effect upon the dehydrogenase and the effect of this concentration on the oxygen uptake of the cells was determined.

Cyanide, (table 4), in a concentration of 1.4×10^{-2} molar greatly inhibited the oxygen uptake of the cells. In general, the inhibition of glucose was less than for the other substances. Since most of the amino acid dehydrogenases

TABLE 4

The per cent increase or decrease in respiration in the presence of potassium cyanide and methylene blue

	POTASSIUM CYANIDE				
	FINAL MOLAR CONCENTRATION			KCN AND M B *	M.B ALONE
	1.4×10^{-2}	1.4×10^{-3}	1.4×10^{-4}		
M. luteus					1 mgm.
Glucose	−70			−45	−3.5
Sucrose	−77			−13	+157
Lactate	−79			−74	+45
Succinate	−95			−94	−6.5
M. flavus					1 mgm.
Glucose	−38			−38	+101
Sucrose	−78			±33	+44
Lactate	−76			−71	+5 1
Succinate	−93			−90	−3 3
M. aurantiacus					0 1 mgm
Glucose			+14	+42	−33
Sucrose			−6.4	−91	−83
Lactate			−12.3	−47	−15
Succinate		−17.1		−76	−63
M. cinnebareus					0.1 mgm
Glucose	−88			−80	−51
Sucrose	−92			−82	−35
Lactate	−93			−71	+57
Succinate			−26	−87	−73
M. freundenreichii					0 5 mgm
Glucose	−77			−73	+25
Sucrose		+9.6		+0 4	+38
Lactate		−25		−63	+39
Succinate	−54			−51	−59

* The cyanide concentration used was that recorded in the first column to the left, and the methylene blue concentration was that amount in milligrams indicated in column to right of this column.

were strongly inhibited by a cyanide concentration as low as 1.4×10^{-5} molar, the effect of cyanide upon the oxygen uptake was not determined. However, the dl-b-phenylalanine dehydrogenase of *M. cinnebareus* was an exception since it was inhibited only five per cent and the oxygen uptake only nine per cent by a 1.4×10^{-5} molar concentration of cyanide.

Influence of methylene blue upon cyanide inhibition

The methods used were the same as for the study of the inhibitors alone except that for this work the methylene blue was made up with the cyanide and the pH of the mixture was adjusted to 7.05. This mixture of cyanide and methylene blue was added to the cells, buffer and saline and allowed to stand 20 minutes before the addition of the substrate. The oxygen uptake was compared with that in the presence of methylene blue alone which was likewise allowed to remain in contact with the cells for 20 minutes before the addition of the substrate.

The organisms varied in their sensitivity toward methylene blue (table 4). *M. luteus* and *M. flavus* were stimulated by the presence of one milligram of methylene blue in the presence of all substrates except succinate. The other organisms were inhibited by this dye concentration. *M. cinnebareus* was most sensitive to methylene blue since 0.1 milligram exerted strong inhibitive action in the presence of all substrates except lactate with which there was stimulation.

Generally, cyanide inhibition was lessened slightly by methylene blue in the presence of glucose, sucrose, and lactate (table 4). The cyanide inhibition was completely removed by methylene blue only in the case of *M. flavus* with sucrose as the substrate and *M. aurantiacus* with glucose as the substrate. The difference shown in the susceptibility toward cyanide and in the ability of methylene blue to act as a carrier indicates that one single mechanism may not operate in the oxidation of the different substrates.

Sodium monochloracetate in a concentration of 1.4×10^{-2} molar generally inhibited the oxidation of all the substrates with the exception of glucose, with which there was stimulation (table 5). Although sodium azide (table 5) was found to be a stronger inhibitor than sodium monochloracetate (table 5), the differences in stimulation and inhibition were quantitative rather than qualitative indicating that the point of attack of both inhibitors might be the same. From the action of the inhibitors it appeared as if a common mechanism might have been operative in the oxidation of glucose, since all organisms acted the same in that there was stimulation by sodium monochloracetate and sodium azide and inhibition by sodium cyanide. The cyanide inhibition, however, was partially removed by the presence of methylene blue.

Possibly two mechanisms may exist for the oxidation of sucrose since with *M. luteus*, *M. flavus* and *M. freundenreichii* the oxidative mechanism was not susceptible to sodium monochloracetate or sodium azide. It was inhibited, however, by sodium cyanide and was not to any great extent reactivated by methylene blue. However, in the case of *M. aurantiacus* and *M. cinnebareus* the oxidative mechanism was susceptible to all inhibitors, and reactivation did not take place in the presence of methylene blue.

The oxidation of lactate was sensitive to all inhibitors used in these experiments. After inactivation by cyanide, lactate oxidation was reactivated by methylene blue only in the case of *M. luteus*, *M. flavus* and *M. cinnebareus*.

Examination of micrococci for the presence of other respiratory enzymes

Polyphenol oxidase is specific in that it will rapidly oxidize the ortho dihydroxy phenols such as catechol. This is used as a basis for the determination of polyphenol oxidase. The oxygen uptake of the cells was determined at 35°C. in the presence of five milligrams of catechol.

TABLE 5

The per cent increase or decrease in respiration in the presence of sodium monochloracetate and sodium azide

	SODIUM MONOCHLORACETATE			SODIUM AZIDE		
	FINAL MOLAR CONCENTRATION					
	1.4×10^{-2}	1.4×10^{-3}	1.4×10^{-5}	1.4×10^{-5}	1.4×10^{-4}	1.4×10^{-5}
M. luteus						
Glucose	+97			+161		
Sucrose	+3.6			+16		
Lactate	−26			−26		
Succinate	−19			−15		
Glycine			−6.5			−42
M. flavus						
Glucose	+6			−4.6		
Sucrose	+9.1			+16		
Lactate	−27			−9		
Succinate	−7.6			−7.6		
M. aurantiacus						
Glucose	+16			+0.2		
Sucrose		−1.8			−3 8	
Lactate		−7.2				− 0.4
Succinate		+8.9			+2 1	
Glycine					+8 8	
M. cinnebareus						
Glucose		+9.2			+2 6	
Sucrose	−50			−39		
Lactate	−44			−35		
Succinate	−54				−14	
dl-b-Phenylalanine			+4.7			
M. freundenreichii						
Glucose	+3.2			−1.3		
Sucrose		−0.4		+4.1	−2 8	
Lactate	−28				−34	
Succinate	+8.5					

In the Warburg vessel were placed 1 ml. cells, 1 ml. M/30 phosphate buffer pH 7.05, 1.1 ml. saline and 0.2 ml. of twenty per cent NaOH to absorb the carbon dioxide evolved. Five milligrams of catechol in 0.5 ml. of water were placed in the side arm and added after allowing 10 minutes for temperature equilibrium. The oxygen uptake was followed for 60 minutes and compared with the oxygen uptake of the cells in the absence of catechol. The results are shown in table 6.

Catalase can be determined by measuring the oxygen given off from peroxide. A blank was run to determine the oxygen uptake by the cells which was added to the oxygen given up by the peroxide to give the total oxygen liberated.

In the Warburg vessel were placed 1 ml. of cells, 1 ml. phosphate buffer pH 7.0, 1.1 ml. saline and 0.2 ml. of 20 per cent NaOH to absorb the carbon dioxide evolved. One-half milliliter of diluted hydrogen peroxide was placed in the side arm of the vessel and was added after allowing 10 minutes for temperature equilibrium. Preliminary trials indicated the amount of peroxide used to be in excess. The respiration measurements were made at 35°C. for 30 minutes. The results are shown in table 7.

TABLE 6

The polyphenol oxidase activity of the micrococci

ORGANISM	Q_{O_2} (N) IN ABSENCE OF CATECHOL	Q_{O_2} (N) in PRESENCE OF 5 MGM. CATECHOL
M. luteus	30.4	59.9
M. flavus	25.5	116.8
M. aurantiacus	26.6	47.6
M. cinnebareus	38.1	185.4
M. freundenreichii	33.9	38.0

TABLE 7

The catalase activity of the micrococci

ORGANISM	C. MM. O_2 UPTAKE IN ABSENCE OF H_2O_2	C MM. O_2 LIBERATED IN 30 MIN./MGM. N
M. luteus	17.9	46.9
M. flavus	17.6	46 8
M. aurantiacus	20.1	80.2
M. cinnebareus	31.0	291.9
M. freundenreichii	18.8	42.1

The cytochromes, or at least cytochrome-c, is oxidized by the enzyme cytochrome oxidase. This oxidase is a hemin compound and therefore is inhibited by carbon monoxide (in the dark) and by cyanide.

Stotz, Sidwell, and Hogness (1938) found that cytochrome-c and the oxidase were involved in the oxidation of both hydroquinone and p-phenylenediamine, but that cytochrome-b, which does not oxidize hydroquinone, could function quite independently of this system in the oxidation of p-phenylenediamine.

The ability of the micrococci to oxidize hydroquinone and p-phenylenediamine was determined as follows: In the Warburg vessel were placed 1 ml. cells, 1 ml. phosphate buffer M/30, pH 7.05, 1.1 ml. saline and 0.2 ml. of twenty per cent NaOH to take up the carbon dioxide evolved. In the side arm was placed 0.5 ml. of hydroquinone or p-phenylenediamine 0.15 molar. The p-phenylenediamine used was the hydrochloride which was adjusted to pH 6.95 immediately before use.

It was found that hydoquinone showed a considerable rate of auto-oxidation and therefore this rate was determined as a blank in each experiment Paraphenylenediamine, on the other hand, showed a very slow rate of auto-oxidation.

Hydroquinone was not oxidized by any of the organisms with the possible exception of *M. freundenreichii* which showed an endogenous respiration $Q_{O_2}(N)$ of 33.9 while in the presence of hydroquinone the $Q_{O_2}(N)$ was 34.3.

In the presence of p-phenylenediamine an increased oxygen uptake was observed. This is shown in table 8.

M. aurantiacus and *M. freundenreichii* were found to be most active in the oxidation of p-phenylenediamine while *M. cinnebareus* oxidized the compound very slowly. Since hydroquinone was not oxidized by any of the organisms, cytochrome-c may be lacking in these cells. The rapid oxidation of p-phenylenediamine might indicate that cytochrome-b is very active since Stotz *et al.* (1938) found that cytochrome-b could act independently of cytochrome-c and cytochrome oxidase in the oxidation of this compound. The absence of

TABLE 8

The oxygen uptake of the micrococci in the presence of para-phenylenediamine

ORGANISM	$Q_{O_2}(N)$ IN ABSENCE OF p-PHENYLENEDIAMINE	$Q_{O_2}(N)$ IN PRESENCE OF p-PHENYLENEDIAMINE
M. luteus	27 2	102.5
M. flavus	23.5	93.1
M. aurantiacus	14.5	181.4
M. cinnebareus	49 0	68.2
M. freundenreichii	30.7	261.3

cytochrome-c would class these organisms with *Staphylococcus aureus*, *albus*, and *citreus* and *Sarcina aurantiaca* which have been found by Frei *et al.* (1934) to contain the cytochrome components a, b, and d.

In this connection, Krampitz and Werkman (1941) found that *Micrococcus lysodeikticus* oxidized p-phenylenediamine but not hydroquinone. It was suggested that the cytochrome-c of this organism had a protein bearer differing from that of beef heart (which oxidizes both) which caused the potential of the former to be negative with respect to hydroquinone and hence inactive.

SUMMARY

The respiratory activities of *Micrococcus luteus*, *Micrococcus flavus*, *Micrococcus aurantiacus*, *Micrococcus cinnebareus* and *Micrococcus freundenreichii* were studied. Under the conditions of these experiments, it was found that when methylene blue was used as the hydrogen acceptor, the substrates found to be most readily activated were raffinose, maltose, sucrose, glucose, ethyl alcohol, succinate, maleate, and glutamate.

When molecular oxygen was used as the hydrogen acceptor the compounds

most readily activated were sucrose, maltose, glucose, ethyl alcohol, succinate, lactate, glutamate, and asparagine.

The oxygen uptake of most of the substrates was constant or decreased slightly with time, while some few of the substrates, mainly glutamate and dl-b-phenylalanine, showed an increasing oxidation rate.

The influence on the cells of several respiratory inhibitors showed that the dehydrogenases of *Micrococcus luteus* and *Micrococcus flavus* were stimulated by a cyanide concentration of 1.4×10^{-2} molar, while the other organisms were inhibited by this concentration. The specific dehydrogenases were found to vary in their sensitivity toward inhibitors. Those which were active against the amino acids were most susceptible in that they were strongly inhibited by sodium azide, sodium monochloracetate and sodium cyanide in a concentration of 1.4×10^{-5} molar.

Cyanide inhibition was generally decreased by methylene blue but was completely removed only in the case of *Micrococcus flavus* with sucrose as the substrate and *Micrococcus aurantiacus* with glucose as the substrate.

Methylene blue when used alone was found to exert an influence on the cells. *Micrococcus luteus* and *Micrococcus flavus* were stimulated by the presence of one milligram of methylene blue in the presence of all substrates except succinate, while the other organisms were inhibited by this amount of methylene blue. *Micrococcus freundenreichii* was stimulated by 0.5 milligram in the presence of all the substrates except succinate while *Micrococcus aurantiacus* and *Micrococcus cinnebareus* were inhibited by a concentration as low as 0.1 milligram.

Sodium monochloracetate in a concentration of 1.4×10^{-2} molar inhibited the oxidation of all substrates with the exception of glucose with which there was stimulation.

With the exception of *Micrococcus freundenreichii* all the organisms possessed moderate polyphenol oxidase and catalase activity.

Hydroquinone was not oxidized by any of the micrococci, while p-phenylenediamine was oxidized by all the organisms. This might indicate that cytochrome-b is abundant in the organisms and that cytochrome-c has a protein bearer which causes its potential to be negative with respect to hydroquinone and hence inactive.

REFERENCES

Braun, H., and Vasarhelyi, J. v. 1932 Ueber die oxydrierenden und reduzierenden Fahigkeiten der Proteusbazillen und über die Beeinflussung dieser Fermentwirkungen durch Antikorper und Antiseptika. Zentr. Bakt. Parasitenk. I. Orig., **127**, 105–111

Callow, A. B. 1924 The oxygen uptake of bacteria. Biochem. J., **18**, 507–519.

Cook, R. P., Haldane, J. B. S., and Mapson, L. W. 1931 The relationship between the respiratory catalysts of *B. coli*. Biochem. J., **25**, 534–550.

Das, N. B. 1937 Identity of lactic and malic dehydrogenases. Biochem. J., **31**, 1116–1123.

Ehrismann, O. 1937 Über Dehydrierenden Leistungen von Mikrokokken und Streptokokken. Z. Hyg. Infectionskrankh., **119**, 572–612.

Fabre, R. 1935 The hydrogen donors for the staphylococci. Compt. rend. **120**, 608–610.

Frei, W., Riedmuller, L., and Almasy, F. 1934 Über Cytochrom und das Atmungssystem der Bakterien. Biochem. Z., **274**, 253–267.

Gale, E. F., and Stephenson, M. 1939 L-malic dehydrogenase and codehydrogenases of *Bact. coli*. Biochem J., **33**, 1245–1256.

Gerard, R. W. 1931 Observations on the metabolism of *Sarcina lutea*. Biol. Bull., **60**, 213–226, 227–241.

Green, D. E., and Brosteaux, J. 1936 The lactic dehydrogenase of animal tissues. Biochem. J., **30**, 1489–1508.

Hawthorne, J. R., and Harrison, D. C. 1939 Cytochrome-C as a carrier with the glucose dehydrogenase system. Biochem. J., **33**, 1573–1579.

Johnson, A. A., and Green, J. R. 1930 Modified methyl red and sodium alizarian sulfonate indicators. J. Ind. Eng. Chem. (Analytical Edition), **2**, 2–5.

Krampitz, L. O., and Werkman, C. H. 1941 Some properties of a bacterial cytochrome system (Abstract) J. Bact., **41**, 21–22.

Krebs, H. A. 1935 Metabolism of amino-acids. III. Deamination of amino-acids. Biochem. J. **29**, 1620–1644.

Stotz, E., Sidwell, A. E., and Hogness, T. R. 1938 The role of the cytochromes in the action of "Indophenol Oxidase". J. Biol. Chem., **124**, 733–744.

Thunberg, T. 1918 Zur Kenntnis der Einwirkung tierischer Gewebe auf Methyleneblau. Skand. Arch. Physiol., **35**, 163–195.

A BACTERIOLOGICAL COMPARISON BETWEEN SYNTHETIC AND NATURAL GLYCEROL[1]

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The superior quality of glycerol synthesized from propylene (Groll, *et al.*, 1938; Williams, 1938, 1941), as compared with glycerol obtained from fats and oils, has been reported by Williams (1938) and confirmed by studies in pharmacology (Hart, 1939) and experiments in histology (Nichols, 1941). Since glycerol is a constituent of numerous culture media for microörganisms, the value of synthetic glycerol as a substitute for natural glycerol would be of interest to microbiologists. In this study the development of certain microörganisms in natural glycerol media was compared with growth in corresponding synthetic glycerol media.

EXPERIMENTAL

In preliminary experiments the utilization of synthetic glycerol (Shell[2]) and natural glycerol (Eastman) was determined by the amount of growth on a balanced salts-agar medium (Allison and Hoover, 1934). After incubation at 30° or 36°C. for 16, 24, and 48 hours, no differences were observed in the extent of growth on the slopes of the synthetic glycerol and natural glycerol media lightly inoculated with washed cells of *Pseudomonas aeruginosa*, *Phytomonas tumefaciens*, *Proteus vulgaris*, *Serratia marcescens*, *Alcaligenes viscosus*, *Achromobacter lipolyticum*, *Staphylococcus aureus*, *Bacillus aterrimus*, *Escherichia coli*, *Aerobacter aerogenes*, and *Mycotorula lipolytica*. Two of the organisms tested, *Alcaligenes lipotylicus* and *Corynebacterium simplex*, both unable to decompose natural glycerol, also failed to utilize synthetic glycerol under the experimental conditions imposed.

EFFECT OF SYNTHETIC GLYCEROL AND NATURAL GLYCEROL ON RESPIRATION OF BACTERIAL CELL SUSPENSIONS

Although the amount of bacterial growth appeared uniform on both kinds of agar media, the possibility remained that the rate of utilization of the synthetic and natural products might vary. The methylene blue reduction technic as well as the Warburg respirometer were employed. As shown in table 1, the dehydrogenase activity of *Escherichia coli*, determined by the method of Isaacs and Nussbaum (1941), remains unchanged whichever type of glycerol substrate is used.

Measurements of oxygen uptake of *E. coli*, *Pseudomonas aeruginosa*, and

[1] Contribution No. 209, Department of Bacteriology, Kansas State College of Agriculture and Applied Science, Manhattan.

[2] Courtesy Shell Development Co., Emeryville, California.

Serratia marcescens were made with the Warburg apparatus, following the general procedure reported by Wilson (1938). The cultures were grown on the high-nitrogen medium 79 of Fred and Waksman (1928) (mannitol omitted) for 16 hours at 28°C., washed with Allison and Hoover's (1934) salt solution, and a suitable mass of cells suspended in M/50 phosphate buffer containing M/50 concentration of natural glycerol or synthetic glycerol. The results shown in table 2 are representative of those obtained in numerous studies with modified experimental conditions.

It is evident from a comparison of the relative rates of dehydrogenation of different sources of glycerol by bacterial suspensions using oxygen and methylene blue as hydrogen acceptors that synthetic glycerol is equal to natural glycerol as a hydrogen donator.

TABLE 1

Methylene blue reduction time of cell suspensions of E. coli with different substrates at 37°C.

SUSPENSION	CONTROL	GLUCOSE	NATURAL GLYCEROL	SYNTHETIC GLYCEROL
	minutes	*minutes*	*minutes*	*minutes*
1	60	10	30	30
2 (diluted)	180	15	75	75

TABLE 2

A comparison of the rate of oxygen consumption (Q_{O_2}) of bacterial suspensions on substrates of natural glycerol and synthetic glycerol

ORGANISM	TEMPERATURE OF RESPIROMETER	Q_{O_2}	
		Natural glycerol	Synthetic glycerol
	°C		
S. marcescens	35	42.3	42.8
P. aeruginosa	35	58.8	58.1
E. coli	35	41.0	37.5
	31.5	40.4	39.3

THE VIRULENCE OF PLANT AND ANIMAL PATHOGENS AFTER GROWTH ON GLYCEROL MEDIA

Cultures of *Phytomonas tumefaciens* were carried on medium 79 with mannitol and the two kinds of glycerol as carbon sources. After frequent transfers at 28°C. over a period of one month, the virulence of young and old cultures was ascertained by testing their ability to induce galls in the stems of the tomato host. Observations at frequent intervals for six weeks revealed no differences in the appearance of galls.

In studies concerning the virulence of *Salmonella pullorum* for baby chicks, cultures were transferred daily for 10 days at 37°C. to beef-infusion broth containing glucose and natural glycerol or synthetic glycerol. The broth cultures

were fed separately to three of four lots of 2-day old chicks from the same pullorum-free brood. Post mortem of chicks for recovery and identification of *Salmonella pullorum* followed established procedures. From the summary of results shown in table 3 it is again apparent that virulence of pathogenic bacteria remains unaltered when synthetic glycerol is a major constituent of culture media.

TABLE 3

Mortality of chicks fed glucose and glycerol broth cultures of Salmonella pullorum

CULTURE	NUMBER OF CHICKS	MORTALITY	
		Total	Positive pullorum
		per cent	*per cent*
Glucose	6	84	80
Natural glycerol	5	100	80
Synthetic glycerol	7	100	86
Water (control)	6	0	0

DISCUSSION

The data confirm previous reports of the desirable properties of synthetic glycerol, and support the belief that it can be as useful in bacteriological culture media as glycerol obtained from biological fats and oils. Synthetic glycerol was not found to be superior to natural glycerol; however, in view of the high degree of purity of synthetic glycerol (Williams, 1938), it may be preferred to the natural product in synthetic media for studies of microbial physiology and certain serological and immunological properties.

SUMMARY

1. Studies of growth on glycerol-salts media and of dehydrogenase activity of bacterial suspensions have shown that synthetic glycerol (Shell) is as good a substrate as glycerol derived from lipids.

2. The cultivation of *Phytomonas tumefaciens* and *Salmonella pullorum* in synthetic glycerol media had no deleterious effect upon the virulence of these bacteria.

REFERENCES

ALLISON, F. E. AND HOOVER, S. R. 1934 An accessory factor for legume nodule bacteria. J. Bact., **27**, 561–81.

FRED, E. B., AND WAKSMAN, S. A. 1928 Laboratory manual of general microbiology. New York, McGraw-Hill.

GROLL, H. P. A., HEARNE, G., BURGIN, J., AND LAFRANCE, D. S. 1938 U. S. Patent 2,130,084. Halo-substitution of unsaturated organic compounds.

HART, E. R. 1939 A pharmacological comparison between synthetic glycerol and glycerol prepared from natural oils. Univ. Calif. Pub. Pharmacol., **1**, 271–4.

ISAACS, M. L., AND NUSSBAUM, M. 1941 A new technic for the determination of specific enzyme systems in bacteria. J. Bact., **41**, 24–25.

Nichols, C. W. 1941 A note on the use of synthetic glycerol (Shell) in the clearing of embryos *in toto*. Stain Tech., **16**, 37–38.

Williams, E. C. 1938 Modern petroleum research. Ind. Eng. Chem., **16**, 630–632.

Williams, E. C. 1941 Synthetic glycerol from petroleum. Trans. Am. Inst. Chem. Engrs., **37**, 157–207.

Wilson, P. W. 1938 Respiratory enzyme systems in symbiotic nitrogen fixation. I. The "resting cell" technique as a method for study of bacterial metabolism. J. Bact., **35**, 601–623.

A MICROSCOPIC ALKALI-SOLUBILITY TEST FOR THE IDENTIFICATION OF GONOCOCCUS COLONIES

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The new procedure described in this paper may be used to identify gonococcus colonies on the primary plate.

The importance of and the methods of culturing *Neisseria gonorrhoeae and Neisseria intracellularis* in diagnosis and in carrier surveys have been indicated (A. P. H. A., 1941). In cultural studies for the diagnosis of gonorrhea the detection of "oxidase-positive" colonies of gram-negative diplococci on the primary plate may be considered sufficient identification of the species in some cases. In medico-legal cases, as well as in chronic gonorrhea in the male or gonorrhea in the female, the species identification by carbohydrate fermentations or alkali-solubility is recommended (A. P. H. A., 1941; Pelouze, 1939; Pitts, 1940; A. M. A., 1937; Knight, 1939). The identification of gonococci by their alkali-solubility was first investigated by Thomson (1923). The identification of meningococcus colonies in either diagnosis or carrier surveys is complicated to an even greater degree by the presence of "oxidase-positive" Neisserias which are normal in the nasopharynx.

The male gonorrhea specimens used in this study were taken from initial and from reinfected cases of acute anterior urethritis and from cases of anteroposterior urethritis. The type of case was determined by the two-glass urine examination (Pelouze, 1939). The female gonorrhea specimens were from cases showing a wide range of clinical symptoms including leukorrhea, pruritis vulvae, frequency, endocervitis, or unilateral bartholinitis. The meningococcus species studied were three strains of lyophiled stock cultures and one strain isolated from a student nurse carrier. The other *Neisseria* species were also cultured from the nasopharynx of students.

METHODS

The specimens were cultured by one of the accepted procedures referred to above (A. P. H. A., 1941). That is, they were streaked on a chocolate agar prepared with Bacto-Hemoglobin and Proteose #3 Agar (Difco Laboratories, 1939) and the plates were incubated in an atmosphere of 10 per cent CO_2 for 48 hours. Isolated colonies of *Neisseria* species were streaked on slants of the same chocolate agar medium in rubber-stoppered test tubes, and the pure cultures so obtained were inoculated into carbohydrate media. These carbohydrate media contained Bacto-Phenol Red broth base, 0.1 per cent agar, 1 per cent carbodrate, and either 10 per cent ascitic fluid or 1 per cent of a chick embryo extract.

The chick embryo extract was prepared by grinding 11-day chick embryos and making a 1 to 5 water dilution of the mash.

The carbohydrates were freshly prepared in 25 per cent solution, autoclaved and added aseptically. Both the chick embryo extract and the ascitic fluid were used either unautoclaved or autoclaved in screw-capped bottles for 20 minutes at 121°C. The carbohydrate media were dispensed in 3 ml. amounts in Wassermann tubes. The cotton plugs in these tubes were cut flush with the mouth of the tube and cork stoppers were inserted for the first 24 hours of the 48-hour incubation period which was generally adequate.

The microscopic alkali-solubility of "oxidase-positive" and of other colonies on the primary plate was determined as follows. Growth from one colony was emulsified as thoroughly as possible in about 0.02 ml. (or 3 loopfuls) of water in the center of a slide. The suspension was so prepared as to show not more than a just perceptible turbidity. A loopful of this suspension was then mixed with two loopfuls of water previously placed on the same slide but to the left of the original suspension. Another loopful of the original suspension was then mixed with a loopful of N/10 or N/5 sodium hydroxide (see results) previously placed on the same slide but to the right of the original suspension. After 30 seconds to 1 minute a loopful of hydrochloric acid of corresponding strength was mixed with the alkali-treated suspension on the right. The three preparations on the slide were then air dried, heat fixed, and stained by Gram's method in the usual manner. The smears were then examined microscopically. The center smear was examined first, to establish the presence of gram-negative diplococci. The smear of the water dilution of the original suspension, that is, the control smear toward the left end of the slide, was examined to determine the normal appearance, degree of autolysis, and the approximate number of organisms before alkali treatment. The smear of the alkali-treated suspension on the right was then examined to determine that the cells were or were not dissolved by the alkali. It was found that when testing soluble Neisserias there was a maximum number of cells which could be dissolved by a given volume of the alkali. Therefore, in the preparation of smears, no more than the cells from one small colony or from part of a large colony were emulsified on the slide.

This microscopic alkali-solubility test was used to determine the primary plate solubility of Neisserias which were isolated and subsequently identified by the described cultural methods. A total of 86 male and 33 female specimens were cultured for the gonococcus, 21 nasopharyngeal specimens were cultured for normal Neisserias, and three strains of lyophiled meningococci were plated.

RESULTS

Fifty-seven of the male specimens and six of the female specimens developed "oxidase-positive" colonies which were tested for N/10 alkali-solubility. All 63 were soluble. Only 33 of the 57 soluble strains from male specimens and 2 of the 6 soluble strains from female specimens were isolated in pure culture and all 35 were found to ferment glucose and not maltose, sucrose, or lactose.

Twenty of the 21 nasopharyngeal specimens developed "oxidase-positive"

colonies. The 20 positive cultures were tested and 19 of the strains were found to resist the action of N/10 alkali. Of these 19 strains which were not soluble in the alkali, 15 fermented glucose, maltose, and sucrose, 2 did not ferment any of the four sugars, and 2 were not tested.

One of the 20 "oxidase-positive" nasopharyngeal cultures fermented glucose and maltose of the four sugars and was soluble in N/10 alkali, and the three meningococcus stock culture strains were found to be partially soluble in the N/10 alkali and definitely soluble in the N/5 alkali. That is, a comparison of the control smear on the left with the alkali-treated smear on the right indicated complete solubility of both the gonococci and the primary isolated meningococci in N/10 alkali, practically complete solubility of the stock culture meningococci in N/5 alkali, and sufficiently complete insolubility of the other Neisserias as to differentiate them.

DISCUSSION

In the preparation of the carbohydrates used to identify *Neisseria*, it was found that autoclaved ascitic fluid was as satisfactory as unautoclaved fluid. This has been previously indicated (Carpenter, 1939) and minimized the hazard of contaminants in the ascitic fluid. The use of chick embryo extract as an enrichment in the carbohydrates was satisfactory if the extract was autoclaved in screw-capped bottles, but was not satisfactory if the sterile extract was not autoclaved before use. Subsequent and current reports of a heat labile-fraction in egg white which inactivates the growth factor biotin (Gyorgy *et al.*, 1941) may or may not bear on this point.

The new test described in this paper should not be recommended as a diagnostic procedure for the identification of meningococcus colonies until it has been further investigated by those examining carriers on a larger scale. As indicated above, there may be a difference in the alkali-solubility of the primary isolated meningococci as compared with the solubility of subcultured strains. Another complicating factor may be found to be the occasional presence of meningococci in genito-urinary infections (Carpenter and Charles, 1941).

On the basis of the work reported here the reliability of the test in identifying gonococcus colonies seems established. If this is confirmed in other hands, the value of the test is obvious. The cultural diagnosis of gonorrhea would involve only the preparation and examination of the primary plates. It would no longer be necessary to subculture well-isolated pure culture colonies for sugar fermentations. That is, a report on the presence of *Neisseria gonorrhoeae* in the specimen could be made within 24 to 48 hours. The "oxidase-positive" colony which is contaminated by other organisms or within a film of spreading growth can be used to determine alkali solubility as satisfactorily as the well-isolated colony. In such cases the contaminating bacteria appear in both the control and in the alkali-treated smear. The test can be performed with a developed "oxidase-positive" colony. Therefore, primary plates on which only one such colony is found may still be the basis for a report of gonococci. There may be gonococcus colonies on primary plates which are not detected because they do not give an

"oxidase-positive" reaction (Pitts, 1940). In such cases the alkali-solubility of suspect colonies should be determined at the time when the gram stain is performed.

The microscopic alkali-solubility method is also under investigation with another objective in mind. That is, we are attempting to identify the gonococcus by treating the gonorrheal exudate or specimen itself with the alkali. If this is possible, the necessity for a cultural examination may be eliminated in the diagnosis of those cases in which the gonococcus is present in the specimen as a morphological entity.

SUMMARY

A new microscopic method is described for differentiating colonies of *Neisseria gonorrhoeae* from colonies of normal flora *Neisseria* species. The differentiation is a function of the relative solubility of the gonococcus cells in a weak sodium hydroxide solution on a slide. This method minimizes the laboratory work and time entailed in rendering a report of *Neisseria gonorrhoeae*. It may also make such reports possible in cases where the Neisserias would otherwise remain unidentified.

REFERENCES

A. M. A. 1937 Queries and minor notes. J. Am. Med. Assoc., **108**, 2157–2158.

A. P. H. A. 1941 Diagnostic procedures and reagents. American Public Health Association.

CARPENTER, C. M. 1939 The cultural method for the diagnosis of gonococcal infection, the gonococcus and gonococcal infection. Publications of the American Association for the Advancement of Science, No. 11. The Science Press.

CARPENTER, CHARLES M., AND CHARLES, RUTH. 1941 The isolation of group 1 meningococci from the genito-urinary tract of nine patients. Paper read at the 70th Annual Meeting of the American Public Health Association, Atlantic City, N. J., October, 1941.

Difco Laboratories. 1939 Manual of dehydrated culture media and reagents. Detroit, Mich.

GYORGY, PAUL, ROSE, CATHARINE S., EAKIN, R. E., SNELL, E. E., AND WILLIAMS, R. J. 1941 Egg-white injury as the result of nonabsorption or inactivation of biotin. Science, **93**, 477–478.

KNIGHT, F. AND SHELANSKI, H. A. 1939 Treatment of Acute Anterior Urethritis with Silver Picrate, Am. Jn. Syphilis, Gonorrhea, Venereal Diseases, **23**, 201–206.

PELOUZE, P. S. 1939 Gonorrhea in the male and female. 3rd ed. Philadelphia: W. B. Saunders Company.

PITTS, ANNE C. 1940 Laboratory diagnosis of gonococcal infections. Am. J. Syphilis Gonorrhea Venereal Diseases, **24**, 184–200.

THOMSON, DAVID. 1923 Gonorrhea. Henry Frowde and Hodder and Stoughton, London.

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THE TECHNIQUE OF QUANTITATIVE CHORIOALLANTOIC VIRUS TITRATION

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Although the quantitative "pock counting" method of titrating a wide variety of viruses on the chorioallantois has been used in this laboratory for the last six years, there has been very little application of the method elsewhere. Schaffer and Enders (1939) studied the inactivation of herpes virus by immune serum along these lines and found the method satisfactory. Nelson (1938) has used the technique in roughly quantitative form for work with vaccinia and variola viruses. Haagen (1940) on the other hand considers that the method is quite unsuited for use as a general method for vaccinia virus titration.

In the course of work on the photodynamic inactivation of herpes and vaccinia viruses with methylene blue we have attempted to improve several aspects of the technique. In essentials our present methods are the same as those described by Burnet in 1936 but attention to details has resulted in a considerable improvement in the membranes obtained.

In our experience there is only one important cause for unsatisfactory results; this is haemorrhage on the inoculated surface of the chorioallantois. A large haemorrhage will cause death of the embryo within 24 hours while smaller ones are almost invariably associated with nonspecific lesions of the ulcer type as described in the 1936 monograph. There seems to be an irregular seasonal variation in the readiness with which haemorrhages occur, the late summer being generally the most unsatisfactory period. There are also small-period fluctuations, some weeks' supplies of eggs giving noticeably more satisfactory results than others. We have not been able to control the occurrence of these variations and can only suggest tentatively that they may be related to the nutrition of the laying hens; the possibility that the level of vitamin K may be responsible for the variation might be worth studying.

Irrespective of the intrinsic character of the eggs supplied, the incidence of haemorrhage is largely determined by the care with which the various manipulations incidental to the inoculation are carried out. Haemorrhage may result from the trauma of a drill cut involving the shell membrane, from the turning in of a pointed angle as the triangle of shell is removed or from damage produced when the slit is made in the shell membrane in the first stage of inoculation. The capillaries from which haemorrhage occurs lie very superficially in direct contact with the inner surface of the shell membrane and haemorrhage may also be produced in the process of separating the chorioallantois and shell membrane. The only important modification of technique which we have recently introduced is designed to minimize damage during separation. This is to draw in a drop of sterile saline to act as a fluid wedge and make the falling

away of the chorioallantois under the influence of suction and gravity proceed more easily and smoothly. Direct examination of the chorioallantois in eggs drilled with a large opening for amniotic inoculations shows that with this technique the chorioallantois separates cleanly with no trace of haemorrhage in at least 80 per cent of instances where the technique has been correctly followed and the eggs are in a satisfactory condition. Using the former technique in which the chorioallantois was drawn away from the shell membrane without the use of saline, a certain amount of haemorrhage is almost invariable.

DETAILED DESCRIPTION OF CURRENT TECHNIQUE

Drilling

A "Vulcarbo" disc No. 8 is used with a dental engine and three cuts through the shell are made to form an equilateral triangle 1.0 to 1.2 cm. along each side. Care should be taken to avoid the slightest damage to the shell membrane and our routine is to make the cut deeper at the angles than at the middle of the sides of the triangle. A minor point of convenience is to place the triangle so that with the air space end of the egg to the right, one side of the triangle runs parallel to the long axis of the egg and the two other sides converge toward the observer. This disposition of the triangle facilitates the reflection of the flap of shell membrane during inoculation. The drilling is completed by making a small cut through the compact layer of the shell over the air space. After drilling, the eggs are returned to the incubator. They should be inoculated within two hours of being drilled. Inflammatory changes develop rapidly in the chorioallantois beneath the drill marks and within less than 24 hours the membrane becomes adherent to the shell membrane at these points.

Inoculation

The equipment used is extremely simple, a plasticine stand to support the egg about 3 cm. above bench level, a straight triangular cutting-edge needle mounted in a suitable handle, a medium-sized nail or some similarly pointed object to make the opening into the air sac, capillary pipettes and rubber teats.

An opening is first made into the air sac with the nail point. With the needle each corner of the triangle is gently prised up a little until it is clear that the triangle of shell will lift off easily. If this precaution is not taken it is easy to lift one side of the triangle and force the sharp angle opposite through the shell membrane. In the middle of the area of shell membrane so exposed a drop of sterile bland fluid (normal saline with 0.044 per cent $CaCl_2$ is usually used) is placed. Through this drop a slit is made in the fibres of the shell membrane and enlarged sufficiently to be sure that the saline has come in contact with the upper surface of the chorioallantois. The details of making the slit are as follows: The needle point should be only moderately sharp and should contact the shell membrane at an angle of about 45°. It is pressed slightly downwards until the point engages the fibres of the shell membrane and then raised so as to split the membrane along the direction of its fibres. .The edge of

the slit should be raised sufficiently to extend the opening about 3 mm. and to allow a glimpse of the chorioallantois. Haemorrhage should be completely absent. The egg is now put aside for a minute or two while others are similarly dealt with. During this period the saline begins the work of gently separating the chorioallantois from the shell membrane. The next stage is to complete the formation of the artificial air space by suction with a rubber teat over the opening into the natural air sac. This should be done very gently and the suction stopped as soon as the chorioallantois is seen to fall away from the shell membrane. Once started, the weight of the egg contents will complete the process.

The slit is now enlarged with the needle and a flap made by cutting with the edge of the needle along the longitudinal edge of the triangle. If this flap of moist shell membrane is turned forward and pressed on to the shell it will adhere and leave a suitable opening for inoculation. The inoculating pipette is a capillary pipette with rubber teat, the capillary having been previously graduated at 0.05 ml. with a weighed drop of mercury. The pipette is held vertically and the inoculum deposited on the chorioallantois without touching shell or shell membrane.

Sealing

After a trial of transparent adhesive tape for sealing the opening we have reverted to the original method of using a coverslip on a rim of vaseline-paraffin mixture. The only technical point worth noting is to avoid allowing the melted paraffin to encroach on the edge of the opening. If the shell membrane—shell junction is infiltrated with paraffin right around the triangle, the air in the artificial air space is absorbed during the subsequent period of incubation.

Removal and examination of membranes

After the required period of incubation the eggs are opened and the chorioallantoic membranes removed. A six-inch petri dish with a suitably moulded pad of wet cotton wool to support the egg is suitable for the purpose. The coverslip is removed and the shell broken away to the level of the fallen chorioallantois. Any shreds of shell membrane are clipped away so that the edge of the area over which the inoculum was spread can be clearly seen. The chorioallantois is now removed with scissors, the cuts being made several millimetres outside the margin of the inoculated area. The membrane is dropped into a dish of 10 per cent formalin in saline and rinsed of blood clot, etc. Then it is spread out on a sheet of black photographic paper kept moist with formol saline. A bent glass spreader is a useful adjunct to the forceps in spreading the membranes satisfactorily. The membranes are left 5 to 10 minutes before being transferred to half petri dishes for examination. After fixation in the stretched condition they will lie flat in saline and the lesions can be examined and counted over a black background. For detailed examination the most convenient set up we have found is to spread the wet membrane out on a square of glass (lantern slide cover) and examine it with a hand lens against a

black background. We have used a petri dish lid painted black as a suitable support and background for examination. A certain amount of light coming in from the side and below makes it much easier to study the membrane than when it is spread directly on a black surface. The larger bloodvessels will usually provide a suitable frame of reference for counting.

In counting the numbers of specific lesions a certain amount of experience of the virus under study is needed in order to become familiar with the various appearances which the lesions may take on different membranes. It is equally necessary to have some experience of the different types of minor nonspecific lesion which may cause difficulty in interpretation. When working with viruses producing small foci the chief features of the specific lesions are (1) an almost circular form, (2) a central more opaque area of necrosis, (3) a surrounding haze due to inflammatory reaction in the mesodermal layer. As a good working rule, if any two of these features are present the lesion can be taken as specific. The differentiation of primary from secondary foci is sometimes difficult and must be based both on the size and distribution of the lesions. With experience it is usually easy to tell from the general appearance of the membrane whether secondary foci are present or not. Where they are numerous it is probably better to make a rough estimate of the number of primary centres rather than to attempt to assess the significance of each specific focus.

In any large series of counts it will be found that the distribution of counts is asymmetrical owing to the occurrence of a proportion of unduly low counts. It is obvious that with a virus every active particle of which is capable of inducing a lesion under optimal conditions, any deviation of the membrane from normal will tend to lower the count. Abnormally high counts beyond the range of random sampling will only be obtained as a result of technical error or a failure to recognize secondary foci as such. Although a rigid proof is difficult it is our impression that with those viruses which are suitable for work on the chorioallantois the count obtained under optimal conditions is very close to the actual number of virus particles present. Low counts are found particularly with two types of membrane, (1) those with a large nonspecific lesion as a result of haemorrhage during inoculation and (2) membranes easily recognized by the almost complete absence of minor nonspecific blemishes in which foci if present are abnormally small as well as unduly low in number. We have been in the habit of referring to these as nonresponsive membranes.

In evaluating the average count from a group of membranes, usually four, inoculated with the same mixture or dilution we have adopted the convention of dividing membranes into those which could reasonably be regarded as giving counts in the normal range and those which for one of several reasons might give counts well outside the standard range. In calculating the average, counts in the first group are given twice the value of those in the second less satisfactory group. The chief reasons for giving the lower value are large nonspecific lesions, nonresponsive type of membrane and inability to be certain of the extent of secondary focus formation. This weighting of counts seems to be the least objectionable way of utilizing personal experience to assess the relative significance of the counts obtained.

Although the relationship between virus concentration and pock count is approximately linear over the range within which reasonably reliable counts can be made, our experience indicates that there is a consistent deviation from strict proportionality. This deviation is in the same direction as that shown in titrations made by analogous methods of plant viruses such as tobacco mosaic (Youden, Beale and Guthrie, 1935) and of bacteriophages (Dreyer and Cambell Renton, 1933). Figure 1 shows the relation between the average, weighted as described above, of two counts from two decimal dilutions in a series of titrations of vaccinia virus. Counts between three and sixteen foci are indicated by dots on the 45° line, while the counts from the corresponding tenfold concentration of virus are shown by crosses at the appropriate position.

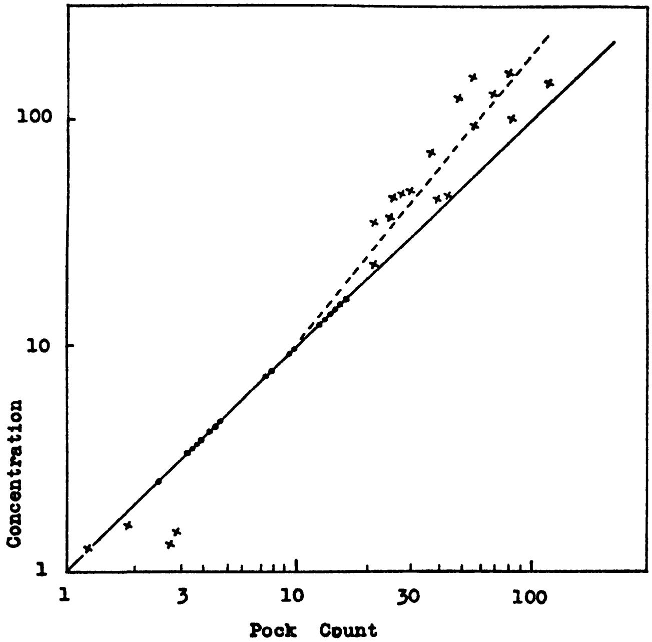


FIG. 1. RELATION BETWEEN VIRUS CONCENTRATION AND POCK COUNT

It will be seen that without exception the counts are lower than would be the case with a strictly linear relationship. For this virus the general nature of the relationship is such as to give a series of the type 1—10—62—300 foci for successive tenfold increases in virus concentration. Wherever possible we prefer to use counts averaging between five and twenty foci for giving a value to the titre of given material. With other viruses the relative diminution in focal counts with increasing concentration is not so evident. With egg-adapted influenza virus strains a simple linear relationship between concentration and count best agrees with the facts.

A detailed titration of a stock of "W.S." egg virus was made primarily to check up the relation between haemorrhage at the time of inoculation and the appearance of the membrane after two days incubation. Dilutions of 1:5000,

1:10,000, 1:20,000, 1:50,000 and 1:100,000 were tested, each on batches of four eggs inoculated through a wide opening to allow a clear view of the initial state of the membrane. Table 1 gives the count recorded for each membrane, the degree of haemorrhage noted at the time of inoculation and the intensity of nonspecific lesions. In this series the averages of the counts fall very close to the values 90—45—22.5—9—4.5 which are proportional to the concentration and there is no indication of any systematic deviation. A glance at the figures

TABLE 1

Titration of influenze virus "W.S. egg" on chorioallantois

A. Relation between virus dilution and focal count

DILUTION	COUNT OF FOCI				WEIGHTED AVERAGE
1:5,000	109	92	88	86	91
1:10,000	50	49	42	40	44
1:20,000	35	25	15	5	23
1:50,000	12	11	11	5	10.5
1:100,000	4	3	2	2	2.75

B. Relation between degree of haemorrhage observed at inoculation and development of nonspecific lesions

DEGREE OF HAEMORRHAGE	NO. OF MEMBRANES	LESIONS AT TWO DAYS							
+	7	++	++	++	+	+	+	+	
±	5	+	±	−	−	−			
−	8	−	−	−	−	−	−	−	−

Under degree of haemorrhage: + signifies easily visible oozing of blood usually giving a patch of at least 1 cm²; ± smaller degrees of visible haemorrhage; − no trace of haemorrhage.

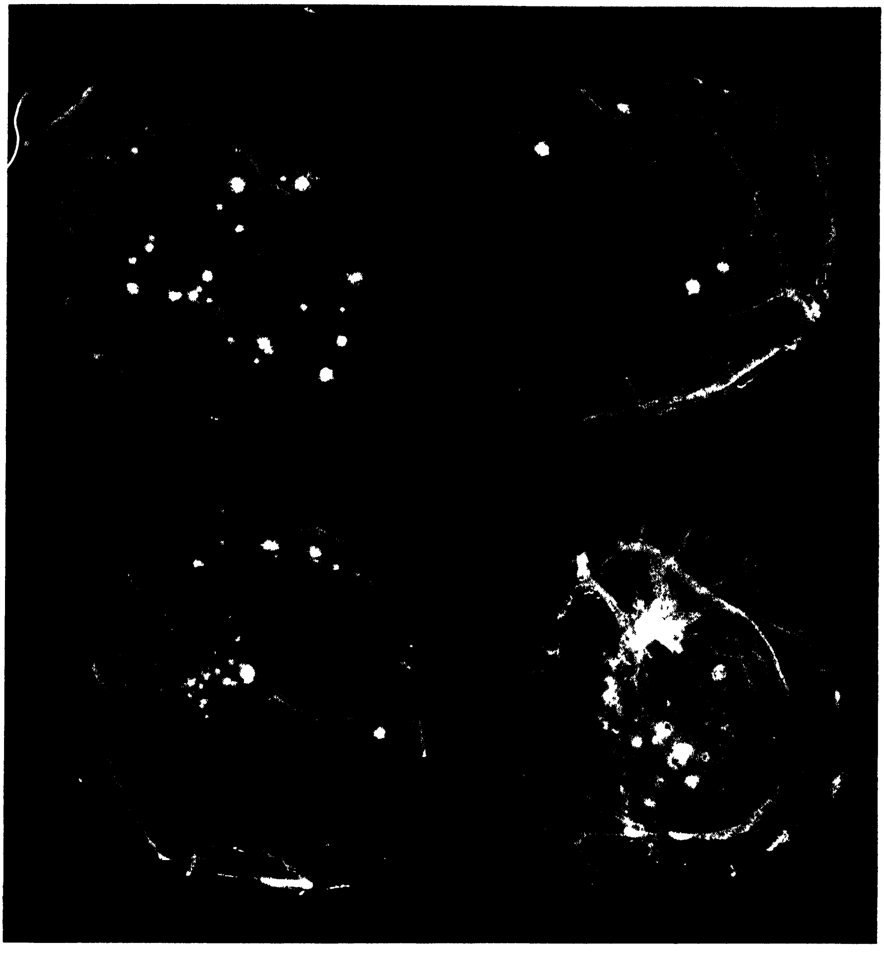
Under nonspecific lesions: +++ (not represented in table) blood stained fluid above membrance with ulcer type nonspecific lesion involving half or more of the inoculated area of the membrane; ++ extensive nonspecific lesion more than 1 sq. cm. in extent; + typical but small nonspecific ulcer; ± minor degrees of nonspecific opacity; − no local traumatic lesions.

will show that the close agreement obtained is better than would normally be expected from the scatter of the counts in each group.

The proportion showing haemorrhage and subsequent nonspecific lesions is unduly high probably on account of the additional manipulation needed with the larger opening in shell and shell membrane. Amongst 22 eggs of the same batch inoculated on the same day by the normal chorioallantoic method one died nonspecifically, two showed +++ lesions, one + lesion and one ± lesions, while the remaining 17 had no nonspecific lesions. There was an absolute correlation between the presence of considerable haemorrhage at the time of inoculation and the appearance of definite nonspecific lesions after incubation.

Preservation of membranes

When it is desired to preserve a membrane as a demonstration specimen it can be mounted in glycerol-gelatin between two lantern slide covers. This method is due to Dr. E. V. Keogh. The membrane is removed from formol

1  2

3 4

FIG 2. FOUR MEMBRANES FROM A SERIES OF VACCINIA VIRUS TITRATIONS: NATURAL SIZE
1 Typical satisfactory membrane with 28 specific foci and slight nonspecific oedema; 2 Technically perfect membrane with 4 specific foci, 3. Membrane showing a patch of secondary foci near the centre and some probable secondary foci in association with primary foci at edge. This membrane was interpreted as having 6 primary foci; 4. Typical severe nonspecific lesion resulting from gross haemorrhage at the time of inoculation.

saline and spread carefully on one of the glass sheets, making sure that there are no bubbles of air beneath the membrane. Excess fluid is drained off and 2 or 3 ml. of melted glycerol-gelatin (gelatin 10 gms., water 60 ml., glycerol 70 ml., phenol 0.25 gm.) placed on the centre of the membrane. The upper glass is then lowered on to this and pressed down carefully to avoid trapping air

bubbles. There is sufficient formalin in the membrane to act as a fixative for the gelatin and an antiseptic. The edges of the preparations are bound as in ordinary lantern slides. The mounted membranes are best examined with oblique illumination against a black background but will also give surprisingly clear pictures when projected on to a screen in ordinary lantern slide fashion.

SUMMARY

The technique of quantitative virus titration of the chorioallantois has been modified to avoid as far as possible the nonspecific lesions produced by haemorrhage when the chorioallantois is separated. A drop of sterile saline is used as a fluid wedge to facilitate this separation. A number of other technical points in the processes of inoculating, removing and counting the membranes are discussed.

REFERENCES

BURNET, F M 1936 The Use of the Developing Egg in Virus Research. Med. Research Council, (Brit), Special Rept Series, No. 220.

DREYER, G , AND CAMPBELL RENTON, M L. 1933 The Quantitative Determination of Bacteriophage Activity and its Application to the Study of the Twort-d'Herelle Phenomenon J. Path Bact , **36:** 399-423.

HAAGEN, H , AND CRODEL, B 1940 Vergleichende Untersuchungen uber die Auswertung von gezüchtetem Variola-Vakzinevirus auf der Chorioallantois des Huhnereies und am Tiere. Zentr. Bakt. Parasitenk , I, ser O, **145:** 249-257

NELSON, J B. 1938 The Behavior of Pox Viruses in the Respiratory Tract I The Response of Mice to the Nasal Instillation of Vaccinia Virus. J. Exptl Med , **68:** 401-411

SHAFFER, M F , AND ENDERS, J F 1939 Quantitative Studies on the Infectivity of the Virus of Herpes Simplex for the Chorioallantoic Membrane of the Chick Embryo, together with Observations on the Inactivation of the Virus by its Specific Antiserum. J. Immunol., **37:** 383-411.

YOUDEN, W. J , BEALE, H. P , AND GUTHRIE, J. D. 1935 Relation of Virus Concentration to the Number of Lesions Produced Contrib Boyce Thompson Inst , **7:** 37-53

NOTES

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ALPHA-NAPHTHOL COLOR TEST FOR DIHYDROXYACETONE AND HYDROXYMALEIC ACID[1]

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In the course of other studies we tested a bacterial culture for acetylmethylcarbinol by Barritt's modification of the Voges-Proskauer reaction (1936). Instead of the expected red, a blue color developed. Suspecting the presence of either glyceraldehyde or dihydroxyacetone, we tested these substances with the same reagent and found that dihydroxyacetone gave a blue color. We have been unable to locate any reference to a similar observation.

The reaction occurs when, to 1 ml. of aqueous solution of the substance to be tested, there is added, first, 0.5 ml. of freshly prepared 6 per cent alcoholic alpha naphthol and, second, 0.2 ml. of 40 per cent potassium hydroxide. In the presence of more than 0.1 milligram of dihydroxyacetone a yellow color soon changes to green and, in the course of a minute or so, this gives way to blue. The blue color is stable for hours. There is no characteristic visible absorption spectrum. The pigment is soluble in polar organic solvents, but not in benzene or ether.

With 1 mgm. pyruvic acid or 25 mgm. acetoacetic ester a blue color also develops. No color is given by glucose, glyceraldehyde, lactic acid, glucuronic acid, acetaldehyde, or acetone. Beta naphthol cannot be substituted for alpha naphthol.

Hydroxymaleic acid, M.P. 144°, prepared according to Wohl and Claussner (1907), yields a pink color which, in concentrations of less than 0.5 mgm. per ml., is slow to develop. With more than 1 mgm. per ml. there is a preliminary formation of the green and blue phases seen with dihydroxyacetone, which pass through purple to a red which has no characteristic visible absorption spectrum. There is no fluorescence of this red substance, in contrast with that formed from diacetyl.

[1] Published with the permission of the Medical Director of the Veterans' Administration, who assumes no responsibility for the views expressed herein

Heating hastens and intensifies the color development. Extraction by amyl alcohol during the blue phase separates the pigment into alcohol-soluble blue and water-soluble red. Extraction later reveals the blue unchanged, but the red gives way to yellow. Late extraction with benzyl alcohol results in all pigment going into the alcohol with a red color.

Conclusion: With alpha naphthol and potassium hydroxide a blue color is given by dihydroxyacetone, pyruvic acid and acetoacetic ester. Hydroxymaleic acid first turns blue, then red.

REFERENCES

Barritt, M.M. 1936 Intensification of Voges-Proskauer reaction by addition of a-naphthol. J. Path. Bact., 42: 441–454.

Wohl, A., and Claussner, P. 1907 Messungen an der Oxymalein- und Oxyfumarsäure. Ber. deut. chem. Ges., 40: 2308–2312.

HYDROGEN IN THE METABOLISM OF AZOTOBACTER[1]

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Phelps and Wilson (1941) found that cultures of *Azotobacter vinelandii* possess an active hydrogenase, the enzyme which activates molecular hydrogen. Because of the possible significance of this finding for nitrogen fixation by *Azotobacter*, we recently tested other species to determine if they too have the enzyme, using O_2 as the hydrogen acceptor according to the technique already described (Wilson, Lee, and Wilson, 1942). The results were:

	A. VINELANDII	A. AGILE	A. CHROOCOCCUM
Q_{O_2} (N)	—	325	118
Q_K (N)	3635	4990	1500
	4365	4180	1500

For this experiment *Azotobacter* cells from 40-hour cultures were used, 0.11 mgm. cellular nitrogen per flask. The Q_{O_2} (N) (mm.^{3} O_2 per hour per mgm. N) was estimated in 96 per cent He, 4 per cent O_2; the Q_K (N) (mm.^{3} total gas uptake per hour per mgm. N), in 96 per cent H_2, 4 per cent O_2. No substrate was added. In this particular trial the hydrogenase activity in *A. chroococcum* was less than in the other two species, but in other experiments it had a Q_K (N) equal to that typical of *A. vinelandii*—about 4000.

The possession of hydrogenase by *Azotobacter* is somewhat unexpected since

[1] Support for this work was furnished in part by grants from the Rockefeller Foundation and the Wisconsin Alumni Research Foundation.

H_2 is not ordinarily concerned in its carbohydrate metabolism which consists primarily in the complete oxidation of the source of carbon to CO_2 and H_2O. Preliminary experiments, however, indicate that suspensions of *A. vinelandii* about 5 to 10 times as heavy as those used for determining respiration or hydrogenase activity induce an anaerobic production of acid and gas from added carbohydrate. The following protocol summarizes our results.

SUBSTRATE	ACID PRODUCTION			GAS PRODUCTION		
	30 min.	90 min.	150 min.	60 min.	120 min.	180 min.
Experiment I						
Glucose	12	22	35	10	21	35
Succinate	10	15	27	17	31	51
———	3	9	18	18	35	54
	30 min.	60 min.	90 min.	60 min.	120 min.	180 min.
Experiment II						
Glucose	18	36	67	12	45	85
Fructose	63	128	226	22	71	140
Hexose diphosphate	46	92	155			
———	2	4	6			

Values are mm.^{3} gas liberated. Acid production estimated in 0.025 M $NaHCO_3$ buffer in gas mixture of 95 per cent He, 5 per cent CO_2; phosphorus in center cup. Gas production determined in phosphate buffer (pH, 7.5) in 100 per cent He; KOH in center cup; gas liberated calculated as H_2. Cellular nitrogen, 0.5 mgm. per flask in each experiment.

The response in some trials was erratic; for example, a lag in gas output would be evident in the presence of both $NaHCO_3$ and phosphate, possibly because of residual O_2 in the gas mixtures. In other experiments, results from duplicate flasks were not too consistent. Nevertheless, the data from the two cited experiments and from others not given suggest that under anaerobic conditions resting suspensions of *Azotobacter vinelandii* can decompose carbohydrate with production of acid and a gas which is probably hydrogen.

REFERENCES

Phelps, A. S., and Wilson, P. W. 1941 Occurrence of hydrogenase in nitrogen-fixing organisms. Proc. Soc. Exptl. Biol. Med., **47**, 473–476.

Wilson, J. B., Lee, S. B., and Wilson, P. W. 1942 Mechanism of biological nitrogen fixation. IX. Properties of hydrogenase in *Azotobacter*. J. Biol. Chem., **144**, 265–271.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH

Iowa State College, Ames, May 8, 1942

Effects of Viruses on Animal Cells. *A. M. Lucas*, Iowa State College.

Histologic Adaptation of the Fox Encephalitis Virus. *R. G. Green and J. C. Barton*, Department of Bacteriology and Immunology, University of Minnesota.

The inclusion bodies of fox encephalitis occur principally in endothelial cells of the cerebral capillary bed. Of 4,723 inclusions in sections of brain, spinal cord, liver, and kidney of 10 naturally-infected foxes, 89% were in cells of the cerebral vascular endothelium and only 2% in the vascular endothelium of the spinal cord, liver and kidney; 7% of the inclusions were in cells of the pia-arachnoidal membrane, and 2% were within nuclei of hepatic cells. There were 27 inclusions per cubic millimeter in brain tissue and only 2 inclusions per cubic millimeter in the spinal cord. In the liver and kidney, also, 2 endothelial inclusions per cubic millimeter were found. In fox encephalitis the presence of an inclusion body evidently indicates growth of virus within the cell, since inclusions appear in ependymal cells only after virus is artificially placed in contact with the cells by inoculation of the cerebro-spinal fluid.

The distribution of inclusions indicates that the fox encephalitis virus is adapted primarily to growth in vascular endothelium and grows secondarily in pia-arachnoidal cells and hepatic cells. This virus does not seem adapted to invasion of nerve cells. The symptoms of encephalitis appear to be caused by destruction of the cerebral vascular endothelium.

Microdiplococci in Filtrates of Natural and Experimental Poliomyelitic Virus as Revealed Unstained by the Electron Microscope and, after Special Staining, by the Light Microscope. *Edward C. Rosenow*, Division of Experimental Bacteriology, Mayo Foundation, Rochester, Minnesota.

During two attempts with the RCA electron microscope microdiplococci, sometimes in short chains, of varying size and opacity, have been found at magnifications of ×12,000, and 10,000, in filtrates (Berkefeld, Seitz and Swinney) of spinal cord from one case of poliomyelitis, of four natural virus strains adapted to monkeys, of five experimental virus strains derived from streptococci and of four chick-mash cultures of streptococci obtained in studies of poliomyelitis. They were not found in respective control filtrates.

After special staining,[1] unmistakable microdiplococci resembling those seen with the electron microscope have been found with the light microscope at magnification of ×1,000 in forty-five filtrates of seven different natural virus strains, in thirty-five filtrates of five different experimental virus strains and in seventy-two filtrates of old chick-mash cultures of twelve different strains of streptococci. Organisms were not found in a comparable number of control filtrates.

The size, shape and density of the microdiplococci were closely similar in the different filtrates but their number varied greatly. The smallest forms revealed by the electron microscope approximated the postulated size of the virus particle. The microdiplococci are considered to be the filtrable or virus phase of the streptococcus which we have isolated consistently in studies of epidemic and experimental poliomyelitis.

Streptococcic Antibody-Antigen Reactions of the Skin and Serum of Macacus Rhesus Monkeys During Attacks of Experimental "Virus" Poliomye-

[1] Proc. Staff Meet., Mayo Clin., 17: 99–106 (Feb. 18) 1942.

LITIS. *Edward C. Rosenow*, Division of Experimental Bacteriology, Mayo Foundation, Rochester, Minnesota.

Intradermal and precipitation tests were made in *Macacus rhesus* monkeys before, during and after attacks of experimental poliomyelitis. Ten per cent solutions of the euglobulin fraction of the serum of horses immunized with streptococci isolated in studies of epidemic and experimental poliomyelitis and other diseases were injected intradermally and precipitation tests were made with the whole antiserums and the serum of monkeys. An immediate edematous cutaneous reaction consistently occurred at the site of injection of the "poliomyelitis" euglobulin and a precipitation reaction was obtained at the interphase between the serum of monkeys and whole poliomyelitis antistreptococcic serum during attacks of poliomyelitis, but not with the respective control euglobulins and whole antiserums. These reactions likewise did not occur in uninoculated monkeys, in monkeys during the period of incubation nor after recovery following inoculation of virus.

Normal monkeys which were given comparable injections of dead streptococci from cases of poliomyelitis and arthritis, respectively, gave positive reactions to the respective homologous euglobulins and whole antiserums.

Intravenous and intramuscular injections of the concentrated poliomyelitis antistreptococcic serum caused the reactivity of skin and serum of monkeys during attacks of poliomyelitis to disappear, whereas injection of control antiserums and normal horse serum did not.

PRELIMINARY SURVEY OF LEPTOSPIROSIS IN MINNESOTA. *Abe Stavitsky*, University of Minnesota, Department of Bacteriology and Immunology.

Using the Schuffner microscopic agglutination test on blood sera, inoculation of young guinea pigs and white mice with blood, urine and tissues, and dark-field examination of these materials, a survey was made of the incidence of leptospirosis in animals in the Twin Cities area and in two groups of the human population there, suspected and confirmed luetics.

Forty-nine humans, consisting of four suspected cases, twenty confirmed luetics, twenty-five suspected luetics, yielded no positives by any method.

Twenty apparently healthy dogs from rural communities near the Twin Cities gave three positive agglutination tests for *Leptospira icterohemorrhagiae*, two in titer of 1:300, one at 1:100, two of these being positive for *Leptospira canicola* at 1:30. One of these dogs also showed a titer for icterohemorrhagiae of 1:100 in its urine. Attempts to isolate leptospirae from blood and urine were negative.

Virulent *Leptospira icterohemorrhagiae*, strains, antigenically similar to strains isolated in other parts of the country, were obtained by animal inoculation of kidney suspensions from two of twenty wild rats caught in the Twin Cities.

Agglutination tests on sera of forty-nine cottontail rabbits and thirteen raccoons were negative, as were attempts to recover spirochetes from a severely jaundiced beaver.

EFFECT OF NITROGEN SOURCE ON APPARENT ACID PRODUCTION FROM CARBON COMPOUNDS BY THE GENUS PSEUDOMONAS. *Richard Bender and Max Levine*, Iowa State College.

Acid production from four monosaccharides by 93 *Pseudomonas* strains isolated from chlorinated water was compared using NH_4Cl and peptone as nitrogen sources. Increasing the incubation period from two to seven days in the presence of NH_4Cl as the sole nitrogen source slightly increased the number of acid-producing strains. With xylose, arabinose and mannose the number of acid-producing strains was three times as great in the synthetic medium as in the presence of beef extract and peptone, and with glucose acid production in the presence of peptone was masked in 35 per cent of the strains indicated as acid-producers in the synthetic medium.

A study of acid production from 13 carbohydrates, 5 polyhydric alcohols and one glucoside showed that 92–96% of the strains produced acid from the monosaccharides, 65–78% from disaccharides, and 58–65% from alcohols. Sixty-seven per cent of the strains produced acid from salicin.

Because of the high percentage of strains fermenting the compounds, acid production from carbon compounds would probably not be satisfactory for the differentiation and classification of the group isolated from chlorinated waters. The need for standardized conditions regarding composition of the basal medium and incubation period in fermentation tests with the group was pointed out.

THE DISINFECTION OF TROUT EGGS CONTAMINATED WITH BACTERIUM SALMONICIDA. *L. L. Gee and N. B. Sarles*, University of Wisconsin.

A COMPARISON OF MEDIA FOR THE DETECTION OF COLI-AEROGENES ORGANISMS IN RAW MILK. *C. E. Skinner and R. M. Marvin*, University of Minnesota.

The numbers of *Escherichia-Aerobacter* organisms in raw milk were determined by means of the dilution-extinction method using standard formate ricinoleate broth and 2% brilliant-green bile broth. No evidence was obtained that one medium repressed more of these bacteria than the other. Gas was produced in a very considerable number of tubes of the formate ricinoleate broth and an insignificant number of tubes of the brilliant-green bile broth from which no lactose fermenters were isolated. The organisms responsible for these false positive tests in the formate broth were largely members of the genus *Proteus*. It is pointed out that *Proteus*, *Salmonella* and other organisms which produce CO_2 and H_2 from glucose are known to produce gas from formate. It is concluded that the inclusion of salts of formic acid in lactose media designed to detect or count *Escherichia-Aerobacter* is a mistake on practical as well as theoretical grounds, inasmuch as gas is produced from formates by certain non-lactose-fermenting bacteria.

OBSERVATIONS ON BACTERIOLOGICAL CONDITION OF CREAMERY WATER SUPPLIES. *H. F. Long and R. T. Corley*, Iowa State College, Ames, Iowa.

Tests on the water supplies of 70 creameries included examination for *Escherichia-Aerobacter* species by enrichment in lactose broth and tryptose lauryl-sulfate broth; total counts on beef extract and tryptone, glucose, extract, milk agars at 37°C.; total, proteolytic and lipolytic counts on tryptone, glucose, extract, milk agar at 21°C.; examination for *Pseudomonas putrefaciens* by culturing on a special gelatin medium after enrichment in litmus milk at 3°C. and washing butter granules churned from thoroughly pasteurized cream with the water and testing keeping qualities of the unsalted butter at 21°C. Approximately half the supplies were unsatisfactory as indicated either by production of defects in unsalted butter or content of *Escherichia-Aerobacter* organisms. Most of the unsatisfactory supplies represented water originally obtained from plant wells although some city supplies were involved. Frequently, water collected directly from the well was satisfactory while that collected at the churn was unsatisfactory, the contamination evidently occurring either in the storage tank or in the piping.

INFLUENCE OF GROWTH AT LOW TEMPERATURE ON HEAT RESISTANCE OF LACTOBACILLUS BULGARICUS. *J. G. Voss and W. C. Frazier*, University of Wisconsin.

Lactobacillus bulgaricus (strain Ga) was carried in sterile reconstituted skim milk at 37.2° and 24.3°C.; transfers were made at 12- and 84-hour intervals, respectively, in order to obtain cultures of an equivalent degree of maturity. During incubation at 37.2° after heat-shocking for 30 minutes at 63°, the 24.3° culture initiated growth more rapidly and gave a more vigorous fermentation than did the culture grown at the higher temperature. Incubation at 47.0° after heat-shocking showed even more striking differences. However, plate counts before and after heat-shocking indicated a significantly greater percentage survival of the 37.2° culture. The fermentation rate at 37.2° of the high-temperature culture after heat-shocking was less than that of the 24.3° culture, although the order was reversed if the organisms were incubated at 37.2° without heat-shocking. The final acidity after heating and incubation, however, was similar in both cases. The data indicate a relatively greater decrease of enzymatic activity in cells of the 37.2° culture on heat-shocking. This might be of

significance in the preparation of starters for Swiss cheese manufacture.

It is evident that measurement of heat resistance by different methods may give conflicting results. The apparent resistance indicated by the degree of activity after heating is at least as significant as that indicated by the decrease in the numbers of viable cells on heating.

OBSERVATIONS ON SOME SOIL CYTOPHAGAS. *W. H. Fuller and A. G. Norman*, Iowa State College.

The aerobic mesophilic cellulose bacteria are less specialized than has been thought. Even among the cytophagas considerable variability and versatility exists.

Three cytophaga cultures, apparently identical with *Spirochaeta cytophaga*, were separately inoculated into cornstalk cellulose suspensions. The xylan present was preferentially attacked and the presence of xylan appeared to render more extensive the utilization of the remainder of the cellulosic material.

Several other organisms identical with the above in morphology, mode of locomotion, and microcyst formation but physiologically considerably less specific have been isolated from soil. One, which does not form microcysts, grows feebly on filter paper but develops vigorously on a wide variety of simple carbohydrates. Some are even incapable of growth on filter paper, but freely use starch and simple sugars. Two such have been isolated and both are morphologically identical with cellulose utilizing forms.

The soil cytophagas are morphologically distinctive and should be separated from other bacteria in any system of classification. However, they exhibit such versatility in physiological characteristics that the inclusion of the requirement "incapable of using carbonaceous materials as food, except cellulose, which is hydrolyzed" in the genus description, cannot be accepted.

HYDROXYLAMINE AS AN INHIBITOR FOR OXIDATIONS BY AZOTOBACTER. *J. B. Wilson and P. W. Wilson*, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin.

Inhibitors have been used to study the mechanism of the Knallgas reaction (oxidation of molecular hydrogen) by *Azotobacter*. Our findings concerning one of these inhibitors, hydroxylamine, have been of particular interest because of the observations by Kubo (Acta Phytochimica, 1937, **10**: 219–238) that this reagent inhibited nitrogen fixation by *Azotobacter*. Kubo's evidence for this consisted of manometric studies with mannitol as the substrate. Using a gas phase of nitrogen and oxygen, he observed that hydroxylamine inhibited gas uptake; whereas, if hydrogen and oxygen were used, hydroxylamine would not inhibit gas uptake.

Our findings indicate that hydroxylamine inhibits the oxidation of organic substrates, but does not inhibit the oxidation of hydrogen. It appears, therefore, that Kubo's observations can be explained on the basis of the inhibition by hydroxylamine of mannitol oxidation and non-inhibition of the Knallgas reaction.

PYRUVIC ACID DISSIMILATION BY CELL-FREE CLOSTRIDIUM BUTYLICUM PREPARATIONS. *H. J. Koepsell and Marvin J. Johnson*, University of Wisconsin.

Preparations obtained by low-temperature vacuum drying of water extracts of frozen *Clostridium butylicum* cells convert pyruvic acid quantitatively into acetic acid, carbon dioxide, and molecular hydrogen. Since the preparations have no hydrogenlyase activity, formic acid cannot be a precursor of the gases. The reaction is most rapid at pH 6.5, where Q_{H_2} values of 60 are obtained. The reaction rate is increased as much as 5-fold by the addition of rat or pork liver kochsaft. Cocarboxylase, Coenzymes I and II, adenine, flavine dinucleotide, riboflavine, adenylic acid, magnesium and manganese will not replace the kochsaft.

Inorganic phosphate is necessary for the reaction. At phosphate concentrations below 0.02 M the reaction rate is proportional to the phosphate concentration. No stable phosphorylation product accumulates.

FIXATION OF CO_2 BY A CELL-FREE EXTRACT OF ESCHERICHIA COLI. *George Kalnitsky and C. H. Werkman*, Iowa State College, Ames, Iowa.

An active, cell-free enzyme preparation has been obtained from *Escherichia coli*, by grinding the organisms with powdered glass and extracting with phosphate buffer. Pyruvate is dissimilated anaerobically to acetic, formic, and succinic acids, carbon dioxide and a trace of lactic acid. No hydrogen is formed. Reaction proceeds at pH range of 6.4 to 7.0; with the optimum at pH 6.8.

The preparation contains strong formic dehydrogenase and hydrogenase activity as determined by methylene-blue reduction. Oxalacetate and fumarate are reduced in an atmosphere of hydrogen. No hydrogenlyase activity is present. In an atmosphere of 10% CO_2 in H_2 with bicarbonate present, CO_2 is fixed, and succinate is formed in amount corresponding quantitatively to the CO_2 uptake.

$C^{13}O_2$ was fixed in formic and succinic acids. The amount of C^{13} in formic acid suggests that most of it was formed from carbon of pyruvic acid and not by reduction of CO_2. The formation of succinic acid from pyruvate and CO_2 with C^{13} in the carboxyl groups is of particular interest because it shows that one can obtain fixation of CO_2 with formation of a carbon to carbon linkage, with a cell-free enzyme system. This offers opportunity for isolation of the enzyme concerned.

FIXATION OF HEAVY CARBON ACETALDEHYDE BY ACTIVE JUICES. *Noel H. Gross, H. G. Wood, and C. H. Werkman*, Iowa State College, Ames, Iowa.

The mechanism of acetylmethylcarbinol formation has been a controversial question for some time. Silverman and Werkman (1940) working with juices prepared from *Aerobacter aerogenes*, did not find any utilization of acetaldehyde, and therefore concluded that acetaldehyde did not participate in the formation of acetylmethylcarbinol. Green *et al.* (1942) observed, with yeast or heart muscle preparation, an increase in acetylmethylcarbinol production when acetaldehyde was added to the pyruvate fermentation and found that other homologous aldehydes gave analogous ketols.

The experiments of the above investigators were repeated using heavy carbon acetaldehyde synthesized by a modification of the method of Cramer and Kistiakowsky (1941).

The fermentation mixtures contained the enzyme preparation, pyruvate, phosphate buffer and heavy carbon aldehyde.

The investigations of both Silverman and Werkman, and those of Green *et al.* have been confirmed. There was no fixation of heavy carbon aldehyde by the bacterial juice; all of the heavy carbon was found in the recovered aldehyde. The yeast juice, however, fixed heavy carbon aldehyde in acetylmethylcarbinol.

BACTERIAL OXIDATION OF 2,3-BUTYLENE GLYCOL. *David Paretsky, H. G. Wood, and C. H. Werkman*, Bacteriology Section, Iowa Agricultural Experiment Station, Ames.

The oxidation of 2,3-butylene glycol by cell suspensions of *Aerobacter indologenes* was investigated. There is an initial rapid rate of oxidation followed by a sharp decrease; the reaction stops completely within 3-4 hours.

Assuming a simple oxidation to acetylmethylcarbinol, only a fraction of the theoretical quantity of oxygen is taken up. The addition of NH_2OH permits the reaction to go to completion with utilization of 100 per cent of the theoretical oxygen. No CO_2 is formed.

Cyanide, azide, iodoacetate, and arsenite are inhibitory in the order given. Cyanide (.01M) gave a 100% inhibition; arsenite (.01M) gave 7%. Variation of pH within a range of 5.46–6.6 has little effect on the reaction, whereas an increase in concentration of organisms tends to force the reaction toward acetylmethylcarbinol.

Acetylmethylcarbinol inhibits the reaction, and its accumulation is perhaps responsible for the cessation of the oxidation of 2,3-butylene glycol.

A FERMENTATION VESSEL FOR THERMOGENIC STUDIES WITH PURE CULTURE. *W. V. Bartholomew and A. G. Norman*, Iowa State College.

The evolution of heat which accompanies the aerobic decomposition of plant materials has been studied in an adiabatic apparatus,

which has recently been improved by the replacement of the central decomposition vessel by one of new design permitting the use of pure cultures. The vessel is suspended by three glass tubes from the lid of a pressure cooker, which in turn is submerged in a bath the temperature of which is controlled by the decomposing material. Access to the vessel is provided by a removable bottom plate through which an air inlet tube passes. After sterilization the vessel is attached to the lid of the pressure cooker and sterile thermocouples for temperature measurement and control inserted down two of the glass tubes. The third serves as an outlet for the air stream. The system is fully insulated and can be operated without contamination.

OHIO BRANCH

COLUMBUS, OHIO, MAY 9, 1942

SPONTANEOUS AND EXPERIMENTAL INFECTIONS IN VITAMIN-B DEFICIENT MONKEYS. *Samuel Saslaw, John L. Schwab, Oram C. Woolpert, and Henry E. Wilson*, Department of Bacteriology, Ohio State University, Columbus, Ohio.

INACTIVATION OF MYXOMA VIRUS BY HEAT. *L. H. Bronson, Jr., and R. F. Parker*, Western Reserve Medical School, Western Reserve University, Cleveland, Ohio.

The virus of infectious myxomatosis of rabbits (in the form of elementary bodies suspended in normal serum) was exposed to heat of varying degree for predetermined intervals of time. It was found that at each temperature the rate of inactivation of virus was constant. With increase of temperature, the rate of inactivation increased regularly: when the logarithm of k was plotted against the temperature the points fell on a straight line.

POLIOMYELITIS IN CUYAHOGA COUNTY, OHIO, IN 1942. *M. Kramer, J. A. Toomey, H. J. Knapp, and J. A. Doull*, Western Reserve University, Cleveland, Ohio.

METHODS OF QUANTITATIVE ESTIMATION OF AIR-BORNE BACTERIA IN ROOMS AND INCUBATORS. *A. R. Winter and G. F. Godfrey*, Department of Poultry Husbandry, Ohio State University, Columbus, Ohio.

A quantitative study of the bacteria in air has been made for the purpose of determining the efficiency of germicidal lights for reducing bacteria in the air of incubators, battery rooms, and food storage rooms. The slit sampler (Bourdillon, *et. al.*: J. Hyg., **41**, 197, 1941) and the bubbler pump (Wheeler, *et. al.*: Sci., **94**, 445, 1941) methods were compared for this purpose. Air was drawn through the apparatus by a suction pump and measured by a gas meter used for calorimetry work.

Higher counts were obtained in most cases by the bubbler pump method than by the slit sampler. This may be due to the fact that the bubbling would have a tendency to separate bacteria from particles carrying them, whereas the sticking of the particles to the agar in the slit sampler would not result in separation. Determinations are easier to make with the slit sampler than with the bubbler pump and the chances of contamination are less. The slit sampler is satisfactory for determining dust particles and bacteria in the air of incubators, battery rooms, and food storage rooms.

MACHINE-ROLLED STOPPERS FOR CULTURE TUBES. *Harold W. Bachelor*, Ohio Agricultural Experiment Station, Wooster, Ohio.

A simple lathe has been constructed which permits the rapid rolling of stoppers for culture tubes. The stoppers can be made of any fibrous material including absorbent and non-absorbent cotton, glass wool, etc. They may be made to any desired dimension, with straight sides or with tapered sides to fit unselected tubes of varying diameters. They may be made solid for use with routine cultures or hollow for use with pipettes. The stoppers possess a definite structure because they are machine rolled and may be used repeatedly. The use of the lathe and of its products is demonstrated.

NUTRITIONAL REQUIREMENTS FOR GROWTH OF LEUCONOSTOC MESENTEROIDES. *Sidney Gaines and Grant L. Stahly*, Department of Bacteriology, Ohio State University, Columbus, Ohio.

Using a medium consisting of acid-hydrolyzed vitamin-free casein, sodium acetate, glucose, cystine-hydrochloride, tryptophane, and six inorganic salts, the vitamin requirements of *Leuconostoc mesenteroides* were investigated. It was found that nicotinic acid, thiamin, pyridoxin, and pantothenic acid had to be supplied to the basal medium before growth of this organism occurred. Due to the fact that traces of biotin were present in some of the constituents of the medium, growth of *L. mesenteroides* was secured when this vitamin was not added as a supplement. Previous work with biotin concentrates had indicated that this organism required biotin for growth. The procedure of avidin inactivation of biotin demonstrated that biotin had to be incorporated in the basal medium before growth of *L. mesenteroides* occurred even though all other necessary factors were present.

Several purine and pyrimidine bases, namely, guanine, uracil, adenine, and xanthine were tested for their growth-promoting or stimulating ability, but were found to be without effect on our strain of *L. mesenteroides*.

VACCINE PROPHYLAXIS AGAINST TULAREMIA IN MAN. *Foshay, L., Hesselbrock, W. H., Wittenberg, H. J., and Rodenberg, A. H.*, Department of Bacteriology, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital, and the Health Center, Department of Health, City of Cincinnati.

Over an eight-year period 2145 persons received prophylactic vaccination against tularemia. For initial vaccination three ½ ml. doses were given on either consecutive or alternate days. The vaccine used was oxidized by nitrous acid.

Evidence for antigenicity of the vaccine was obtained by agglutination tests performed with serums from vaccinated individuals obtained after vaccination, after revaccination, and one year after vaccination. Revaccination provoked titers that were lower than those seen after initial vaccination, although revaccinated persons were better protected.

During the nine-year period of study there were 14 cases with no deaths among 2145 vaccinated persons while there were 357 cases and 27 deaths in the general population from which the experimental group was derived. The authors point out that no inference can be drawn as to the relative degree of protection conferred in the vaccinated group since the number of exposed individuals in the general population group is unknown. More convincing evidence of protection was obtained from a study of vaccinated individuals who acquired tularemia. There was striking amelioration of all measurable aspects of the disease such as duration of disability, fever, adenopathy, bed-ridden period, and length of disease.

IMMUNOLOGIC SPECIFICITY OF A HISTAMINE-AZO-PROTEIN CONJUGATE. *Sanford B. Rosenman and W. A. Starin*, Department of Bacteriology, Ohio State University, Columbus.

During a study of the efficacy of histamine compounds, and their respective antiserums in the prevention of anaphylactic shock, we have synthesized the p-aminobenzoyl derivative of the amine. Following diazotization the compound was combined with various serums (human, horse and bovine). The resultant product resembled the histamine-azo-protein described recently by Sheldon, Fell, *et. al.*; the antigen of the latter workers was coupled to despeciated horse-serum globulin. Prolonged immunization of rabbits resulted in the production of relatively low titer serum as demonstrated by the presence of precipitins by the ordinary ring test method and by the collodion agglutination technic. Cross reactions occurred when the test antigen consisted of the haptene coupled to other proteins. The precipitation reactions were inhibited partially or completely by the addition of p-aminobenzoylhistamine to the immune serum. The fact that the protein had not been completely altered was demonstrated by the cross reactions which occurred with the native serum. Specific complement-fixing antibodies were also shown to be present.

CENTRAL NEW YORK BRANCH

CORNELL UNIVERSITY, MAY 16, 1942

FACTORS AFFECTING THE PRODUCTION OF FORMIC ACID BY STREPTOCOCCI. *I. C. Gunsalus and C. F. Niven, Jr.*, College of Agriculture, Cornell University, Ithaca, N. Y.

Data which came to our attention suggested the possibility of a relationship between the reaction and the yield of volatile acids during the fermentation of glucose by homofermentative streptococci.

To determine whether this were the case, fermentation balances at different reactions were run on growing cultures of *Streptococcus liquefaciens* in a tryptone, yeast extract, phosphate-buffered medium containing glucose as the fermentable substrate.

When the reaction during fermentation was held to a pH of approximately 5, the lactic acid formed accounted for over 90 per cent of the glucose fermented. Only traces of volatile acids were produced. On the other hand, when the fermentation occurred at pH 9.0, the lactic acid accounted for 60 per cent or less of the glucose fermented while formic and acetic acids and ethyl alcohol, in the molecular ratio of 2:1:1, accounted for about 30 per cent. At reactions intermediate between pH 5 and pH 9 the proportion of lactic acid and volatile products varied between these limits. A sharp decrease in the volatile products occurred below pH 6.5.

The concentration of protein in the medium has a definite, though slight, effect on the yield of volatile acids.

THE NUTRITIVE REQUIREMENTS OF THE "VIRIDANS" STREPTOCOCCI WITH SPECIAL REFERENCE TO STREPTOCOCCUS SALIVARIUS. *C. F. Niven, Jr. and K. L. Smiley*, College of Agriculture, Cornell University, Ithaca, N. Y.

A medium consisting of chemically defined components has been devised which will support growth of *Streptococcus salivarius* for an indefinite number of transfers. The simplest medium contained the following substances: inorganic salts, glucose and sodium thioglycolate; d-glutamic acid, dl-leucine, dl-iso-leucine, d-arginine, dl-lysine, dl-methionine, l-tyrosine; riboflavin, nicotinic acid, calcium pantothenate, thiamin and biotin methyl ester. Uracil, though not essential, was found to increase the rate of growth to such an extent that maximal growth was obtained within 24 hours.

The vitamin requirements for *Streptococcus salivarius* differ from those of Lancefield groups B and D, reported by Woolley and Hutchings, in that *Streptococcus salivarius* requires nicotinic acid, thiamin, and biotin, and does not need vitamin B_6. Tryptophane, an indispensable amino acid for group D organisms, was not needed by *Streptococcus salivarius*.

Several members of each of the recognized species and serological groups of streptococci were tested for growth in the synthetic medium described above. None was found to be able to initiate growth in the medium except two strains of *Streptococcus bovis*. Upon investigation of the vitamin requirements of one of these, it was found that only thiamin, nicotinic acid, and biotin were needed; riboflavin was not required.

NASOPHARYNGEAL CULTURES IN THE DIAGNOSIS OF WHOOPING COUGH. *Anne M. Brooks and W. L. Bradford*, University of Rochester, School of Medicine and Dentistry, Rochester, N Y.

The bacteriological diagnosis of whooping cough is usually made by the cough-plate method. In young children, it is difficult to obtain satisfactory plates, since the cough is frequently more "hacking" than paroxysmal. In these cases, a method of nasopharyngeal culture has proved useful.

A flexible, cotton-tipped wire is inserted far back into the nasopharynx through the naris. The swab is then streaked on Bordet-Gengou medium, and the plate is examined for *Hemophilus pertussis* after a 48-hour incubation.

When the swab cannot be streaked immediately it is stored in a rubber-stoppered test-tube containing a piece of dampened rubber sponge. *H. pertussis* survives for as long as 48 hours under these conditions.

In children less than three years old, the nasopharyngeal culture was positive for *H.*

pertussis in 57.5 per cent of 106 cultures; the cough-plate, in only 25.5 per cent.

With children of all ages and at all weeks of the disease, 61 per cent of 248 primary nasopharyngeal cultures were positive in contrast to 37 per cent of 157 primary cough-plate cultures.

Of 248 cases of whooping cough, 85 per cent were diagnosed bacteriologically by either single or repeated nasopharyngeal cultures.

THE ROLE OF p-AMINOBENZOATE IN EXPERIMENTALLY DEVELOPED SULFANILAMIDE-FASTNESS IN THE GONOCOCCUS. *H. E. Stokinger, R. C. Charles, and C. M. Carpenter, and C. M. Carpenter,* University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

p-Aminobenzoate (PAB), in addition to being a growth stimulant for certain bacteria, exerts pronounced antisulfonamide activity. These facts suggested that sulfanilamide-resistance of bacteria might depend upon increased formation of PAB.

Analyses for substances of the type of PAB (primary aromatic amines) were made on broth cultures of four experimentally developed sulfanilamide-fast strains of the gonococcus and of the corresponding non-resistant parent strains. The analytical method consisted in diazotization, followed by coupling with thymol to produce colored derivatives. The results indicated that the sulfanilamide-fast variants produced increased amounts of PAB or related substances. On the other hand, addition to the medium of azochloramid, an inhibitor of the antisulfonamide activity of PAB, failed to lower the resistance of the drug-fast strains to sulfanilamide. Assuming that any PAB present was available to the action of azochloramid, the latter result indicated that PAB was not involved in sulfanilamide-fastness in the gonococcus.

Data were also obtained on the growth-stimulating and antisulfanilamide effects on the gonococcus, of PAB and the narcotics, procaine and urethane. PAB was the most potent in both respects. Procaine, closely related chemically to PAB and sulfanilamide, exerted greater antisulfanilamide activity than urethane, but approximately equal growth-stimulating action.

AN AZOLITMIN-LIKE PIGMENT PRODUCED BY CERTAIN ACTINOMYCETES. *Jean E. Conn,* New York State Agricultural Experiment Station, Geneva, N. Y.

In a paper presented at an earlier meeting (Conn and Conn, J. Bact., **39**, 21, 1940), mention was made of several strains of *Actinomycetes*, which possessed pigments having indicator properties. A further study of two of these species has yielded data indicating that the pigment produced by them, when extracted with dilute NaOH, bears a close resemblance to azolitmin, which has always been regarded as the active principle of litmus. The superficial appearance of the two is very similar, since both are blue in an alkaline solution, turning red and precipitating when acid is added. To pursue this point further, absorption curves have been made for both the pigment and azolitmin at different pH-values. These curves show a marked resemblance in their absorption maxima, especially in alkaline solution.

Since litmus is no longer available, an extensive search is being made for a substitute, which can be used in milk. Synthetic azolitmin has been prepared, but has not as yet proved satisfactory. Experiments are now under way to determine whether the pigment produced by these *Actinomycetes* can possibly be used as a substitute for litmus.

VARIATIONS OF THE STANDARD CURVE IN THE MICROBIOLOGICAL ASSAY OF RIBOFLAVIN. *F. W. Tanner, Jr.,* N. Y. State Agricultural Experiment Station, Geneva.

THE RIBOFLAVIN REQUIREMENTS OF CERTAIN STRAINS OF LACTIC-ACID-PRODUCING ORGANISMS. *Thressa E. Campbell,* N. Y. State Agricultural Experiment Station, Geneva.

EFFECTS OF AZOCHLORAMID AND SULFONAMIDES IN LOCALIZED INFECTIONS OF MAN. *Erwin Neter,* University of Buffalo and Children's Hospital, Buffalo, N. Y.

The synergistic antibacterial action of sulfonamides and azochloramid *in vitro*, and the observation of Schmelkes and Wyss of an inhibitory action of azochloramid upon para-aminobenzoic acid (antisulfonamide)

prompted a study on the effects of these compounds in human infections. A case of wound infection, caused by a hemolytic, coagulase-positive strain of *Staphylococcus aureus*, was unsuccessfully treated with sulfanilamide ointment, sulfathiazol ointment, sulfathiazol by mouth, and sulfadiazine powder locally. Application of azochloramid-sulfanilamide solution resulted in the disappearance of the staphylococcus, even for 8 days after this treatment was discontinued. Later, staphylococci were again recovered. In a case of empyema caused by *Streptococcus hemolyticus*, treatment by mouth with sulfadiazine and sulfanilamide failed to reduce the number of viable streptococci. The exudate contained approximately 5,000,000 streptococci per ml., despite the presence of free sulfanilamide in concentrations ranging from 9.2 to 12.65 mg. per cent. Intrapleural administration of azochloramid-sulfanilamide solution reduced the number of viable streptococci to 2,400 per ml. within 24 hours. From then on, the number of streptococci decreased steadily and, following a second injection of the azochloramid-sulfanilamide solution, the fluid became permanently sterile. The possible applications of the synergistic action of azochloramid and sulfonamides are discussed.

Application of Legume Bacteria to Chemically Treated Pea Seed. *A. W. Hofer and G. L. McNew*, New York State Agricultural Experiment Station, Geneva, N. Y.

In recent years, it has been found that seed decay of certain vegetables could be greatly lessened by chemical treatment of the seed previous to planting. The compounds used for this purpose are cuprous oxide, organic mercury compounds, and tetrachloroparabenzoquinone (commercially designated as Spergon). Treatment of seed of legumes such as peas, with which the use of chemicals has been most successful, raises a serious question in regard to inoculation of such seed with nodule bacteria.

A series of laboratory and greenhouse tests has been made to determine the effect of the different protectants on the nodule organism for peas. All of the seed protectants were found to be lethal in concentrated dosage, but sufficient bacteria survived for 30 minutes on Spergon-treated seed to produce effective inoculation. The other protectants destroyed practically all cells of *Rhizobium leguminosarum*, as shown by dilutions of washings from the seed, and by nodulation of plants in the greenhouse.

Inoculation of Spergon-treated seed was most successful when low concentrations of chemical (1–1½ oz. per bu. of seed) were used, and the number of bacteria was increased beyond the recommended dosage. Further tests are in progress to determine whether successful inoculation of Spergon-treated seed can also be secured under field conditions.

The Composition of Gas from Experimental Silos. *J. K. Wilson*, Department of Agronomy, Cornell University, Ithaca.

MARYLAND BRANCH

Baltimore, June 4, 1942

Intramammary Injection of Homogenized Oil-Sulfanilamide in the Treatment of Bovine Mastitis. *J. C. Kakavas*, Department of Bacteriology and Hygiene, University of Delaware, Newark, Delaware.

The oral administration of sulfanilamide has been unsuccessful for the treatment of bovine mastitis. However, when sulfanilamide is injected in large amounts directly into the udder through the teat canal, the mastitis streptococci are readily destroyed in the mammary tissue. It was found that sulfanilamide could be introduced into the udder of a cow very readily by properly suspending the drug in light liquid petrolatum. One pound of sulfanilamide is added to 900 cm^3 of light liquid petrolatum and mixed thoroughly by means of a mechanical mixer. The mixture is then passed through a homogenizer, resulting in a thin uniform creamy suspension which remains stable. One ml. of the homogenized oil-sulfanilamide contains 0.378 gram of sulfanilamide.

In streptococcic mastitis the dosage recommended for an average size udder is 40 cm^3 of the homogenized product given daily for four days. If the infection persists, the treatment may be repeated with safety. In staphylococcic mastitis the dose recommended is 80 cm^3 of the product given daily for four days and repeated if indicated. The homogenized oil-sulfanilamide, as used in these experiments was well tolerated and no contra-indications have been manifested in the cases treated. There was no appreciable decrease in milk production during or after treatment. One hundred and three cows were treated for streptococcic mastitis by intramammary injections with homogenized oil-sulfanilamide. One hundred of these were infected with *Streptococcus agalactiae* and three with *Streptococcus uberis*. Based on bacteriological findings, cures were effected in 92 cows or 89.3 per cent, and partial or no improvement in 11 cows or 10.7 per cent. Breaking down the results into quarters treated, of the total 265 quarters, 251 or 94.7 per cent were cured, and 14 or 5.3 per cent were not cured.

THE ISOLATION AND ABSORPTION SPECTRUM MAXIMA OF BACTERIAL CAROTENOID PIGMENTS

BEN SOBIN AND GRANT L. STAHLY

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The production of pigments by bacteria has attracted attention from the beginning of the study of bacteriology, possibly because the presence of pigment is one of the characteristics of microorganisms which is most readily observed. In recent years special emphasis has been placed on the chemical nature of the pigments and on their probable rôles in the metabolism of bacterial cells.

The bacterial pigments whose chemical structures have been determined fall into four main groups (White, 1939). Prodigiosin, the red pigment of *Serratia marcescens*, is a pyrrole derivative. Pyocyanin, the dark blue pigment of *Pseudomonas aeruginosa*, was the first phenazine derivative to be found in nature. Phthiocol, isolated from the tubercle bacillus, is a naphthaquinone derivative. However, the largest group of bacterial pigments belongs to the class of compounds known as carotenoids. Most yellow, orange, and red pigmented bacteria contain carotenoids. This interesting group of compounds occurs extensively throughout both the plant and the animal kingdoms. In the plant world the carotenoids are present in forms ranging from bacteria to the highest plants. Similarly, in the animal kingdom we find these compounds in many forms of both invertebrates and vertebrates.

Research on the carotenoids in plants and animals has resulted in the development of techniques whereby these compounds can be separated and identified. Because of the ease of obtaining large amounts of material, considerable knowledge of the carotenoids in plants has been gained as evidenced by such monographs as those of Palmer (1922) and Strain (1938). The investigation of the carotenoids present in bacteria has lagged, possibly, because of the greater difficulties of obtaining sufficient material.

The presence of carotenoids in bacteria was demonstrated by Zopf (1889) who designated this group of bacterial pigments, lipochromes, because of their solubility in the fat solvents. Later, Kligler (1914) and Krainsky (1914) demonstrated the widespread presence of carotenoid pigments in different species of bacteria.

Only a few of the large number of bacteria known to contain carotenoids have been subjected to pigment identification. Reader (1925) found beta-carotene and lycopene in *Sarcina aurantiaca* and a hitherto unreported pigment, which she named coralin, in *Streptothrix corallinus*. Chargaff and Dieryck (1932) reported the presence of a xanthophyll and a hydrocarbon, which they called sarcinene, in *Sarcina lutea;* Nakamura (1936) found a xanthophyll ester in this bacterium. Chargaff (1933) found zeaxanthin to be the only pigment in *Staphylococcus aureus* and differed with Reader (1925) in reporting beta-carotene and

zeaxanthin in *Sarcina aurantiaca*. Chargaff (1933) isolated beta- and gamma-carotenes and Ingraham and Steenbock (1935) isolated alpha- and beta-carotenes, cryptoxanthin, and esters of lutein, zeaxanthin, and azafrin from *Mycobacterium phlei*. Two additional acid-fast bacteria were studied by Chargaff and Lederer (1935). *Bacillus lombardo-pelligrini* contained beta- and gamma-carotenes and the bacillus of Grassberger was found to contain beta- and gamma-carotenes, and lycopene.

The carotenoid pigments in some purple sulphur bacteria were investigated by Karrer and Solmssen (1936). Rhodoviolascin, rhodopin, rhodopurpurin, flavorhodin, and rhodovibrin were isolated and described briefly. Probably most of these pigments are limited in their occurrence to the sulphur bacteria.

A carotenoid pigment named spirillo-xanthin was isolated from *Spirillum rubrum* by van Niel and Smith (1935). Leprotin, a carotinoid hydrocarbon, was found by Grundmann and Takeda (1937) in an acid-fast bacterium isolated from a leprous lesion.

The carotenoid pigments most commonly found in bacteria belong to the following groups: (1) hydrocarbons, such as beta-carotene; (2) alcohols, such as xanthophyll; (3) esters; and (4) carotenoid acids.

It is clear that more information is desirable concerning the chemical nature of the bacterial carotenoid pigments. Such knowledge would be an aid in the solution of fundamental problems, such as the role of these pigments in bacterial metabolism. Although the structure and certain other characteristics of some of these pigments have been established, data on other pigments are lacking. The isolation and spectrometric analysis of the carotenoids are basic preliminary steps in their investigation. An aspect of the present study has been the adaptation of the methods used for the separation and identification of the carotenoid pigments of higher plants to the bacterial carotenoids. Absorption spectrum maxima have been determined and compared with those recorded in the literature for similar pigments. Complete identification of the pigments which were isolated is not claimed but pertinent data are presented.

MATERIALS AND METHODS

Cultivation of the bacteria. The pigmented bacteria were grown on nutrient agar containing two per cent glycerol and adjusted to pH 7.2. Inoculations were made by spraying the surface of the medium with a twenty-four-hour broth culture, using an atomizer through which sterile air was passed. Sixteen-ounce French square bottles were used for culturing the bacteria. After incubation for one week at room temperature, the cells were removed by the addition of a 70 per cent aqueous solution of acetone and the scraping of the surface with a glass rod bent at a right angle. Water was not used because it frequently forms a hydrophilic suspension with the cells from which the latter are removed with difficulty. The pigments were not extracted by the aqueous acetone and the bacterial cells were separated by centrifugation.

Extraction of pigments. Kuhn and Brockmann (1932) employed absolute methanol for extracting the carotenoid pigments from finely divided plant

material. The present investigators found that cold methanol was effective in extracting the carotenoids from moist bacterial cells when the mixture was ground with an abrasive such as alundum. A convenient method of extraction involved the addition of 50 ml. of methanol to approximately one gram of moist bacterial cells and the placing of the container in hot water to bring the methanol to the boiling point quickly. The pigments were extracted in a few minutes. The solution was then cooled and the cells removed by centrifugation. The short exposure to an increased temperature was found to produce no injurious effects on the pigments.

Separation of types of pigments. The methanol extract then was subjected to partition between immiscible solvents to separate the various types of carotenoid pigment. A modification of the method of Kuhn and Brockmann (1932) is represented schematically in figure 1.

Add water to the methanol extract to give an alcohol concentration of 90 per cent. Shake in separatory funnel with petroleum ether.

- Petroleum ether phase
  (Contains hydrocarbons and esters.) Evaporate to dryness. Saponify with alcoholic potassium hydroxide. Add water to make alcohol concentration 90 percent. Shake with petroleum ether.
  - Petroleum ether phase
    (Contains hydrocarbons.)
  - Methanol phase
    (Contains freed alcohols.)
- Methanol phase
  (Contains alcohols and carotenoid acids.) Make alkaline, dilute with water, and shake with diethyl ether.
  - Ether phase
    (Contains alcohols.)
  - Aqueous phase
    Acidify and extract free carotenoid acids with diethyl ether.

FIG. 1. SCHEME OF SEPARATION OF BACTERIAL CAROTENOID PIGMENTS

The methanol extract was diluted with water to give an alcohol concentration of 90 per cent and then was shaken in a small separatory funnel with an equal volume of petroleum ether. The petroleum ether layer was drawn off and shaken several times with fresh portions of ninety per cent methanol. Similarly, the alcohol layer was shaken repeatedly with petroleum ether in order to insure a complete separation of the pigments.

The petroleum ether layer was freed from water by shaking with saturated salt solution and standing over anhydrous sodium sulphate for half an hour, and then was evaporated to dryness under partial vacuum. The dried pigment may contain hydrocarbons and esters. Fifty milliliters of a two-and-one-half-per cent solution of potassium hydroxide in methanol was added and the solution was kept at 40°C. for three hours. Water was then added to give an alcohol concentration of 90 per cent and the solution was shaken in a separatory funnel with petroleum ether.

The petroleum ether layer was drawn off, washed repeatedly, freed from water, and evaporated to dryness under reduced pressure. The residue was taken up in petroleum ether and the individual hydrocarbons were then separated by chromatographic analysis.

The freed carotenols in the alkaline methanol layer were forced into diethyl ether by the addition of half-saturated salt solution. The ether layer was washed with water until the washings were no longer alkaline to phenolphthalein, freed from water, and then evaporated to dryness under vacuum.

The original methanol phase containing carotenols and carotenoid acids was made alkaline to litmus, diluted with water, and shaken with diethyl ether. The ether portion was washed, freed from water, and evaporated to dryness.

If there are any carotenoid acids present, they remain in the aqueous phase in the form of their salts. The acids may be recovered by the addition of dilute hydrochloric acid and extraction with diethyl ether.

The procedures just described cover all four of the main types of carotenoid pigments; certain steps are eliminated if partition tests reveal the absence of any of these groups.

Chromatographic adsorption. After separation of the pigments into groups, the individual components in each group were separated by the chromatographic adsorption technic devised by Tswett (1906) and excellently described by Cook (1936). Zechmeister and Cholnoky (1937) have also presented a thorough description of chromatographic adsorption.

Successful separation of the carotenoids is dependent on the selection of the proper adsorbent and solvent and on the careful preparation of the adsorption column.

The choice of adsorbent and solvent depends largely upon the particular group of carotenoids to be separated. The hydrocarbons have a weak affinity for adsorbing substances; hence, a strongly adsorbing material must be employed. Strain (1938) recommended for the separation of leaf xanthophylls a mixture of a specially prepared magnesia[1] with an equal amount of heat-treated siliceous earth[2]. Bacterial carotenols were too strongly adsorbed on this mixture but it proved to be entirely satisfactory for the separation of the carotenoid hydrocarbons, when either petroleum ether or ethylene chloride was employed as the solvent.

Calcium carbonate, activated by heating at 150°C. for five hours, followed by cooling in a vacuum desiccator, served well for the separation of the carotenoid alcohols. Petroleum ether and carbon disulphide are the solvents of choice for the carotenols.

Calcium hydroxide and a mixture of aluminum oxide and siliceous earth were tested as absorbents but were found to be less satisfactory than those described.

The adsorption column must be prepared carefully in order to insure satisfactory separation of the pigments. The adsorption device used in this investi-

[1] Micron Brand, magnesium oxide No. 2641, California Chemical Company, Newark, California.

[2] Hyflo super cel F.A. 501, Johns-Manville Co., New York.

gation consisted of a glass tube, 15 cm. long and 15 mm. in diameter, sealed at one end to a tube of 6 mm. bore and approximately 8 cm. in length. The tube was supported in a vertical position by attachment to a vacuum flask. A wad of cotton was placed just above the constricted portion of the tube. The adsorbent was added in small portions, each of which was packed with a rod to which a metal disk, slightly smaller than the diameter of the tube, was attached. One of the solvents was poured onto the column and suction was used to test for the presence of cracks in the column.

Certain precautionary measures must be observed in chromatographic adsorption. The pigments must be free of water before they are dissolved in the adsorbing solvent. Small amounts of moisture may be removed by adding benzene and evaporating *in vacuo* at 50°C. The presence of a trace of ethanol also interferes markedly with the adsorption.

The pigment mixture was dissolved in about 10 ml. of solvent and the solution then was poured onto the adsorption column so that the latter was covered quickly. As the solution passed through the column the pigments formed a narrow band near the top. The column then was washed with fresh portions of pure solvent which caused the adsorbed pigments to move slowly through the adsorbent. If filtration is slow, gentle suction may be applied. The pigments are separated gradually into a series of bands each of which represents a distinct pigment. When several zones were present, continued washing with the solvent washed through those which were adsorbed weakly. The latter were collected separately in the suction flask. The zones remaining in the column were separated mechanically and eluted with ethanol and then filtered from the adsorbent, using a mat of siliceous earth.

In addition to its use for the separation of similar pigments, chromatographic adsorption is useful for pigment identification. If a solution of an unknown pigment is mixed with that of a known pigment and adsorbed on a column, the formation of a single colored zone indicates that the two pigments are very similar or identical. The formation of two zones proves that the two pigments are not identical. Similarly, if two pigments found to possess identical or nearly identical absorption maxima by spectrometric analysis are suspected of being the same pigment, they can be tested by mixing and adsorbing them on a column. The formation of a single band indicates their unity; the formation of two zones proves their dissimilarity.

Spectrometric analysis. The carotenoid pigments are distinguished conveniently by their absorption spectra. There are several instruments available for obtaining such spectra. In this investigation a Bausch and Lomb spectrometer, equipped with a constant deviation prism of the Pellin-Broca type, was used. At a sufficiently low concentration of pigment, the bands of maximum absorption are symmetrical enough to allow one to determine the mean value of their boundaries by placing the crossed hairs of the instrument over the darkest portion of the band. The value so obtained was recorded as the absorption maximum for each band.

The absorption spectrum maxima of the carotenoids were determined in 95 per cent ethanol, in carbon disulphide, and in chloroform.

A Baly tube was used for varying the amount of solution through which the light passed. A photographic flood lamp was used as the light source. The spectrometer was calibrated with the sodium D line and checked periodically with this standard. The absorption maxima obtained were compared with those recorded in the literature for various carotenoids dissolved in the same solvents.

TABLE 1
Bacterial cultures used

NAME	CULTURE NUMBER	SOURCE
Flavobacterium arborescens	435	American Type Culture Collection
F. suaveolens	958	American Type Culture Collection
F. esteroaromaticum		Cornell University
F. sulphureum	42.70	Ohio State University
F. fecale		Cornell University
Sarcina lutea	8.40	Ohio State University
S. flava	147	American Type Culture Collection
S. aurantiaca	146	American Type Culture Collection
Micrococcus luteus	379	American Type Culture Collection
M. flavus	400	American Type Culture Collection
Erwinia lathyri		National Type Culture Collection, England
E. ananas		Malaya—Dr. Perry Elrod
Bacterium mycoides	35.10	Ohio State University
Cellulomonas flavigena	482	American Type Culture Collection
Staphylococcus aureus	209	U.S.D.A. Phenol Coefficient Test Strain
Staphylococcus aureus	610	Food poisoning—Ohio State University
Staphylococcus aureus	614	
Staphylococcus aureus	615	
Staphylococcus aureus	616	
Staphylococcus aureus	617	
Staphylococcus aureus	620	
Staphylococcus aureus	626	
Staphylococcus aureus	628	Throat infection—Ohio State University
Staphylococcus aureus	628a	Animal infection—Ohio State University
Staphylococcus aureus	628b	Furuncle—Ohio State University
Staphylococcus aureus	628c	

Bacterial cultures. The pigmented bacteria used in this study are listed in table 1.

EXPERIMENTAL

The absorption maxima, as determined by spectrometric analysis, for 12 different pigments are recorded in table 2. The pigments were isolated from 14 different species of bacteria and consist of 7 carotenols and 5 hydrocarbons. No esters or carotenoid acids were isolated from these bacteria. The number of the pigments, as referred to in table 2, is arbitrary and is used to identify the several pigments.

Flavobacterium. Inspection of table 2 shows that the single carotenol pigments isolated from *F. esteroaromaticum*, *F. suaveolens*, and *F. fecale* had the same absorption spectrum maxima. Additional evidence for their identity was obtained by subjecting a mixture of the three pigments to chromatographic adsorption on a column of calcium carbonate. A single pigmented zone was obtained. A search of the literature failed to reveal any carotenol possessing the same absorption maxima as the pigment isolated from these three species of *Flavobacterium.*

TABLE 2.

Absorption maxima in different solvents of pigments isolated from various bacteria.

BACTERIUM	TYPE OF CAROTENOID	NO. OF PIGMENT	COLOR AND ZONE IN ADSORPTION COLUMN	ABSORPTION MAXIMA (MU)								
				CS_2			$CHCl_3$			C_2H_5OH		
Flavobacterium esteroaromaticum	Alcohol	1	Orange	453	482	513	460		488	452		482
F. suaveolens	Alcohol	1	Orange	453	483	514	459		488	451		482
F. fecale	Alcohol	1	Orange	453	482	513	459		488	451		483
F. sulphureum	Hydrocarbon	2	Yellow	437	466	499	451		481	440		470
F. arborescens	Hydrocarbon	3	Zone 1—orange	465	498	536	450	480	516	440	470	503
	Hydrocarbon	4	Zone 2—red	495	530	572	477	508	546	463	494	530
	Hydrocarbon	5	Zone 3—orange	465	500	540	450	480	515	439	469	503
	Hydrocarbon	2	Zone 4—yellow	435	465	499	450		480	439		469
	Hydrocarbon	6	Zone 5—orange	465	500	540	450	480	515	439	469	503
Sarcina lutea	Alcohol	7	Zone 1—yellow	460		496	451		480	439		470
	Alcohol	8	Zone 2—yellow	464		499	450		480	440		470
S. flava	Alcohol	7	Yellow	460		495	450		480	440		470
S. aurantiaca	Alcohol	9	Orange	479		514	463		498	456		487
Micrococcus luteus	Alcohol	7	Zone 1—yellow	460		495	451		480	440		470
	Alcohol	8	Zone 2—yellow	465		499	450		480	440		470
M. flavus	Alcohol	7	Yellow	459		494	449		480	439		469
Erwinia lathyri	Alcohol	10	Yellow	478		513	458		485	452		483
E. ananas	Alcohol	11	Yellow	474		508	460		493	450		480
Bacterium mycoides	Alcohol	12	Red	477	508	548	454	487	521	446	474	506
Cellulomonas flavigena	Alcohol	7	Zone 1—yellow	460		496	451		480	440		470
	Alcohol	8	Zone 2—yellow	464		499	450		480	440		470

A single carotenoid hydrocarbon was obtained from *F. sulphureum*. A strongly adsorbed yellow zone was obtained with the specially prepared magnesium oxide mixture. Chargaff and Dieryck (1932) obtained a hydrocarbon from *Sarcina lutea* which had the same absorption maxima when dissolved in petroleum ether as has this pigment. They named their pigment sarcinene. Whether the two pigments are identical is not certain since none of Chargaff's pigment was available for chromatographic analysis.

The delicacy of chromatographic adsorption is exemplified by the separation of the pigments of *F. arborescens*. Partition experiments showed the presence of hydrocarbons only. Five distinct zones were obtained on the magnesium oxide mixture when ethylene chloride was used as the solvent. The third and fifth zones had identical absorption maxima in the three solvents. The orange

pigment from the first zone was also very similar. The three zones, however, were separated from each other on the adsorption column by distinctly different pigments. When these three pigments were mixed and adsorbed on a column of magnesium oxide, three zones were obtained, thus indicating their individuality. Molisch (1914) obtained a pigment from a sulphur bacterium which he called alpha-bacteriopurpurin. He determined its absorption maxima in carbon disulphide only; the values were the same as those obtained for the red pigment from zone 2. Perhaps, the yellow pigment from the fourth zone is the sarcinene of Chargaff and Dieryck.

When *F. arborescens* was grown on a medium containing glycerol or glucose, a red pigmentation was obtained; when grown on nutrient agar, an orange growth was produced. Pigment analysis, however, indicated only a quantitative difference; the same five hydrocarbons were identified. The amount of the red pigment was increased by growth on media containing glucose or glycerol.

Sarcina. Two carotenoid alcohols were obtained from *S. lutea.* As noted before, Chargaff and Dieryck reported a hydrocarbon which they called sarcinene in this species. A second strain of *S. lutea* was investigated in this laboratory and yielded the same two carotenols but no hydrocarbon. It is interesting to note (table 2) that the absorption maxima were almost identical in chloroform and in ethanol. When the two pigments were mixed and readsorbed on calcium carbonate, two zones were obtained again. Pigments with these absorption maxima have not been reported previously.

S. flava contained a single carotenol which apparently was identical with one of the pigments of *S. lutea* as indicated by absorption maxima and adsorption of the mixed pigments.

S. aurantiaca contained a single carotenol which was unreported previously.

Micrococcus. Two carotenols were found in *M. luteus.* Absorption maxima and chromatographic adsorption with mixed pigments indicated that these were the same as the alcohols obtained from *Sarcina lutea.* The two species of bacteria were differentiated, however, by their fermentation reactions.

A single carotenol was isolated from *M. flavus.* It apparently was the same pigment as one of those found in *S. lutea, S. flava,* and *M. luteus.*

Erwinia. Single and distinct carotenoid alcohols, both unreported previously, were isolated from *E. lathyri* and *E. ananas.*

Bacterium mycoides. This organism is not to be confused with *Bacillus mycoides.* An intense red pigmentation was obtained which was due to a single red carotenol. A pigment, isolated by Karrer and Solmssen (1935) from a purple sulphur bacterium and named by them rhodopin exhibited the same absorption maxima.

Cellulomonas flavigena. This bacterium produced two carotenols. Absorption maxima and mixed chromatographic adsorption indicated that these pigments were identical with those obtained from *S. lutea* and *M. luteus.*

Staphylococcus aureus. Twelve strains of *S. aureus* obtained from various sources and with different histories were subjected to pigment analysis. Table 3 presents pertinent data. All the strains examined were found to contain a

hydrocarbon whose adsorption maxima coincided with delta-carotene reported by Winterstein (1933) to occur in the fruit hulls of *Gonocaryum pyriforme*, and also a carotenoid alcohol whose absorption maxima were identical with rubixanthin isolated by Kuhn and Grundman (1934) from rose hips. In addition to these two pigments, four strains isolated from food poisoning outbreaks and four strains from staphylococcic infections contained an ester of rubixanthin.

The strain of *S. aureus* used by the Food and Drug Administration of the U. S. Department of Agriculture as the test organism for determining phenol coefficients contained, as a third pigment, a hydrocarbon with absorption maxima identical with those of sarcinene. Chargaff (1933) reported that zeaxanthin

TABLE 3

Pigments isolated from various strains of Staphylococcus aureus

STRAIN	SOURCE	PIGMENTS		
		Hydrocarbon	Alcohol	Third pigment
209	Food and Drug Administration	*Delta-carotene	†Rubixanthin	‡Hydrocarbon-sarcinene
614	Food poisoning	Delta-carotene	Rubixanthin	†Ester of rubixanthin
615	Food poisoning	Delta-carotene	Rubixanthin	Ester of rubixanthin
616	Food poisoning	Delta-carotene	Rubixanthin	
617	Food poisoning	Delta-carotene	Rubixanthin	
620	Food poisoning	Delta-carotene	Rubixanthin	
626	Food poisoning	Delta-carotene	Rubixanthin	†Ester of rubixanthin
628	Infection	Delta-carotene	Rubixanthin	Ester of rubixanthin
628a	Infection	Delta-carotene	Rubixanthin	Ester of rubixanthin
628b	Infection	Delta-carotene	Rubixanthin	Ester of rubixanthin
628c	Infection	Delta-carotene	Rubixanthin	Ester of rubixanthin

* This pigment had absorption maxima identical with those of delta-carotene.

† Same absorption maxima as for rubixanthin.

‡ Same absorption maxima as for Chargaff and Dieryck's sarcinene.

was the only pigment in *S. aureus*. In none of the twelve strains studied was this pigment isolated.

DISCUSSION

It has been an important feature of this investigation to adapt some of the methods used for the separation and identification of the carotenoid pigments found in plant material to the separation and the determination of absorption spectrum maxima of the carotenoid pigments present in bacteria. It was found possible to make with ease a pigment analysis starting with approximately one gram of moist cells.

One of the problems encountered in this study was the extraction of the carotenoids from the bacteria. The extraction of carotenoids from plant material may be accomplished by the use of any one of many of the fat solvents. This was not true for the bacterial carotenoids. It was discovered that the

extraction of the carotenoids from the bacterial cells could be accomplished best in the presence of a small amount of water with a water-miscible fat solvent. Methyl alcohol proved to be well suited for this purpose.

Formerly, when carotenoid pigments were isolated they were given names, often without regard to their chemical structures. Sometimes, the name signified the source, e.g. sarcinene from *Sarcing lutea* and violacein from *Chromobacterium violaceum*. More recently, as new pigments have been isolated, names have commonly been withheld until the chemical structure of the pigments could be determined. This appears to be a rational policy, especially since source names lose their significance when the same pigment is found in different kinds of plants or animals.

The present investigation resulted in the isolation of several carotenoid pigments with absorption maxima previously unreported. Some bacteria were found to contain but one pigment while others contained several. With the exception of those in *S. aureus*, the carotenoid pigments in any one species of bacteria belonged to a single type, with the alcohols predominating. No carotenoid acids were found in any bacterium.

Several of the different species of bacteria contained identical pigments. An interesting problem arises as to whether pigment analysis should be included in securing characteristics for differentiation of bacteria. Consistency of pigment production is important in this respect. It has been found during the course of this study that each bacterium produced the same pigments repeatedly. When *F. arborescens* was grown on a medium containing glycerol or glucose, a red pigmentation resulted; on nutrient agar, an orange growth was obtained. However, upon pigment analysis the same five hydrocarbons were obtained in each instance with the amount of red pigment enhanced when the organism was grown on glycerol or glucose. Data are too few, however, to conclude that media have no effect on the kinds of pigment formed by a bacterium.

It is probable that different strains of a certain species of bacterium will be found to produce different pigments. Reader (1925), Chargaff (1933) and the present authors disagree on the pigment formed by *S. aurantiaca*. Likewise, Nakamura (1936), Chargaff and Dieryck (1932) and the present authors fail to agree with respect to the pigments of *S. lutea*. In order to test the hypothesis that different strains of one species produce different pigments when grown on a standard medium, the present investigators subjected twelve strains of *S. aureus* to pigment analysis. Two pigments were common to all, with some variation in regard to a third pigment. Again these results do not coincide with those of Chargaff (1933). Insufficient data on the subject are available to attempt a correlation of pigment type with other properties or with source.

There are interesting opportunities for research in determining the effects of environmental factors on the kinds and amounts of the carotenoid pigments produced by bacteria. Rapid growth makes possible a large number of studies in a short time, the environment may be controlled fairly well, and complicating factors are fewer, in general, than they are for the higher plants. For the above reasons, it appears also that bacteria offer definite possibilities in connection with

discovering the true function, if any, of the carotenoids in plants and animals. Two principal types of reactions have been attributed to the yellow pigments in leaves. One involves chemical reactions taking place in photosynthesis; the other is concerned with various oxidation-reduction reactions in which hydrogen is transferred, presumably by the pigments acting reversibly as hydrogen acceptors and donors. However, careful consideration of the evidence leads to the conclusion that the subject is still in need of much critical investigation.

It is known that some naturally occurring pigments, such as riboflavin, cytochrome, pyocyanine, phthiocol and toxoflavin function as hydrogen transporters in bacterial metabolism. It is evident that studies should be extended to investigations of the functional role of the carotenoids in bacteria with the ultimate purpose of discovering their role in plants and animals in general.

SUMMARY

Methods for the extraction, isolation, and determination of absorption spectrum maxima of bacterial carotenoid pigments have been devised or adapted from those applicable to the higher plant pigments. The pigments were extracted from bacterial cells with hot methanol. They were separated by chromatographic adsorption and then subjection to spectrometric analysis.

Twelve carotenoid pigments were isolated from 14 different species of bacteria. These included five species of *Flavobacterium*, three of *Sarcina*, two of *Micrococcus*, two of *Erwinia*, one of *Bacterium*, and one of *Cellulomonas*. Seven of the carotenoids were alcohols and five were hydrocarbons. Some bacteria produced only one pigment while others produced several; *Flavobacterium arborescens* contained five distinct hydrocarbon carotenoids. Some species which can be separated by fermentation reactions produced identical pigment.

Twelve strains of *Staphylococcus aureus*, obtained from various sources, were subjected to pigment analysis. Pigments whose absorption maxima were identical with those of delta-carotene and rubixanthin were found in all the strains studied. The strain used as a test organism for determining phenol coefficients also contained another hydrocarbon. Four strains isolated from food poisoning cases and four from staphylococcic infections also contained an ester of a carotenol with absorption maxima the same as those of rubixanthin.

It is apparent that bacteria offer interesting possibilities for discovering the function of the carotenoid pigments in nature.

REFERENCES

Chargaff, E., and Dieryck, J. 1932 Die Pigmente der *Sarcina lutea*. Naturwissenschaften, **20**, 872–873.

Chargaff, E. 1933 Sur les carotenoides des bacteries. Compt. rend., **197**, 946–948.

Chargaff, E., and Lederer, E. 1935 Sur les pigments carotenoides de deux bacteries acido-resistantes. Ann. inst. Pasteur, **54**, 383–388.

Cook, A. H. 1936 Chromatographic analysis. Chemistry and Industry, **14**, 724–726.

Grundmann, C., and Takeda, Y. 1937 Ein neues Bakterien-Carotenoid, Leprotin, Naturwissenschaften, **25**, 27.

Ingraham, M. A., and Steenbock, H. 1935 The relation of microorganisms to carotenoids and vitamin A. II. The production of carotenoids by *Mycobacterium phlei*. Biochem. J., **29**, 2553–2562.

KARRER, P., AND SOLMSSEN, U. 1935 Die Carotinoide der Purpurbakterien I. Helv. Chim. Acta, **18**, 1306–1315.

KARRER, P., AND SOLMSSEN, U. 1936 Die Carotinoide der Purpurbakterien II. Helv. Chim Acta, **19**, 3–5.

KLIGLER, I. J. 1914 A biochemical study of certain bacterial pigments. Columbia Univ. Biochem. Bull., **3**, 458–460.

KRAINSKY, A. 1914 Die Aktinomyceten und ihre Bedeutung in der Natur. Zentr. Bakt. Parasitenk., II, **41**, 649–688.

KUHN, R., AND GRUNDMANN, C. 1934 Uber Rubixanthin, ein neues Xanthophyll der Formel $C_{40}H_{56}O$. Ber. deut. chem. Ges. **67**, 339–344.

KUHN, R., AND. BROCKMANN, H. 1932 Bestimmung von Carotinoiden. Z. physiol. Chem. **206**, 41–64

MOLISCH, H. 1914 Die Purpurbakterien. Fischer. Jena.

NAKAMURA, T. 1936 A new bacterioxanthophyll, the yellow pigment of *Sarcina lutea*. Bull. Chem. Soc. Japan, **11**, 176–179.

PALMER, L. S. 1922 Carotenoids and Related Pigments. New York, The Chemical Catalog Company, Inc.

READER, V. 1925 Note on the lipochromes present in certain bacteria. Biochem. J., **19**, 1039–1046.

STRAIN, H. H. 1938 Leaf Xanthophylls. Washington, Carnegie Institute of Washington.

TSWEET, M. 1906 Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. Ber. deut. botan. Ges., **24**, 384–393.

VAN NIEL, C. B., AND SMITH, J. H. C. 1935 Studies on the pigments of the purple bacteria. I. On spirilloxanthin, a component of the pigment complex of *Spirillum rubrum*. Arch. Mikrobiol., **6**, 219–229.

WHITE, D. E. 1939 The chemistry of fungus and bacterial pigments. Australian Chem. Inst. J. and Proc., **6**, 313–330.

WINTERSTEIN, A. 1933 Uber ein Vorkommen von Gamma-carotin. Z. physiol. Chem., **219**, 249–252.

ZECHMEISTER, L., AND CHOLNOKY, L. 1937 Die chromatographische Adsorptionsmethode; Grundlagen, Methodik, Anwendungen. Julius Springer.

ZOPF, W. 1889 Ueber Pilzfarbstoffe. Botan. Ztg., **47**, 53–61.

QUANTITATIVE DETERMINATION OF THE BACTERIOSTATIC EFFECT OF THE SULFONAMIDE DRUGS ON PNEUMOCOCCI

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Determination of the susceptibility of various microorganisms to the bacteriostatic action of the sulfonamide drugs requires a test which is relatively simple to perform, clear and easy to read, and with which readily reproducible results can be obtained. From the clinical standpoint an *in vitro* test has certain advantages as compared with *in vivo* methods. Attention has been drawn by a number of investigators to certain of the factors affecting *in vitro* tests with this group of drugs. Among the variables may be mentioned the nature of the medium employed (Long and Bliss, 1939), the character of the fluid used in making dilutions for the inoculum, the age of the culture (Finkelstone-Sayliss, Paine and Palruk, 1937; Green, 1940; and Woods and Fildes, 1940) and the size of the inoculum (Colebrook, Buttle and O'Meara, 1936; Nitti, Bovet and Depierre, 1937; Domagk, 1937; Rosenthal, 1937; Stamp, 1939; and Fleming, 1940).

The present paper deals with several sources of error in the procedures commonly used and describes an *in vitro* method which, in the studies thus far made, has yielded consistent results in the determination of the relative susceptibility of certain bacteria to the action of the sulfonamide drugs. The results obtained by the use of this technique for testing the relative susceptibility to sulfapyridine of different strains of the disease-producing types of pneumococci will be presented in a subsequent paper.

DILUTING FLUID

The fluid used for diluting the culture of pneumococcus for the proper inoculum is important. It must not of itself be injurious to the bacterium and furthermore must contain no sulfonamide inhibitor. Diluting the culture in water, physiological saline or solutions of buffers either kills some of the cells or so injures them that a prolonged lag phase may occur. If plain peptone broth is used, sufficient inhibitor may be introduced along with the inoculum so as to alter the end point of the bacteriostatic test. The most satisfactory diluting fluid has been found to be an inhibitor-free liver infusion containing 2 per cent rabbit serum. This medium is described in a subsequent section of the present paper.

AGE OF CULTURES USED FOR INOCULATION

Several investigators have reported that the age of the culture used as an inoculum effects the outcome of the bacteriostatic tests. It has been stated that young cultures, during the logarithmic phase of growth, are more susceptible to the action of the sulfonamide drugs than are the cells from older cultures. The reported difference in the susceptibility of young and old cells to sulfon-

amide drugs parallels older observations on the lower resistance of young cells to various other unfavorable agents. For example, in 1910 Schultz and Ritz showed that cells of *Escherichia coli* during the lag phase and in the early logarithmic phase of growth are much more susceptible to heat than in the late logarithmic phase. This observation has been repeatedly confirmed (Winslow and Walker, 1939). Sherman and Albus (1923) reported the greater resistance of older cells of *E. coli* and *Proteus vulgaris* not only to heating but to chilling, hypertonic solutions of sodium chloride and phenol. The greater susceptibility of young cells of *E. coli* to distilled water has been described by Hershey (1939) and to ultraviolet light by Gates (1929–30). In addition, Bayne-Jones and Sandholzer (1933) have demonstrated that young cells are more readily attacked by bacteriophage than are the cells from older cultures.

Variations in the susceptibility of young and old bacterial cells to the action of the sulfonamide drugs may be related to the content of sulfonamide inhibitor in cultures of different ages, and may also be due to variations in the organisms themselves. The presence of sulfonamide inhibitors in bacterial cultures has been demonstrated in a number of laboratories (Stamp, 1939; Green, 1940; Woods and Fildes, 1940; Fleming, 1940; MacLeod, 1940; and MacLeod and Mirick, 1941). The presence of these substances unquestionably affects the outcome of the bacteriostatic tests.

In the present studies, 8- to 10-hour cultures grown in charcoal-adsorbed peptone broth containing defibrinated rabbit blood have been used. At this period of growth cultures of pneumococcus are at the peak of the logarithmic phase. Reproducible results have been obtained with cultures of this age.

SIZE OF THE INOCULUM

A concentration of a sulfonamide drug which is bacteriostatic for a small inoculum of a particular microorganism may have little or no effect if a larger inoculum is used for seeding (Colebrook, Buttle and O'Meara, 1936; Nitti, Bovet and Depierre, 1937; Domagk, 1937; Rosenthal, 1937; Stamp, 1939; Fleming, 1940). However, because of the variations in the amount of sulfonamide inhibitor present in different lots of peptone broth it has not previously been possible to determine accurately the effect of varying the size of the inoculum. When sulfapyridine was used in routine bacteriostatic tests with pneumococci of various types it was found that the results were usually the same whether an inoculum of 10^{-4} ml. or 10^{-5} ml. of culture was used, and the end points were never more widely separated than a two-fold dilution of the drug. These inocula fall within the range of 40,000 to 3,000 cells as determined by colony counts of poured plates. Safe limits are 5,000 to 20,000 cells. Since identical results were almost invariably obtained within these limits it was possible to simplify the method of preparing the dilutions.

A 2 mm. loopful of a fully grown 8- to 10-hour blood-broth culture of pneumococcus is placed in 5 ml. of liver infusion. The suspension is mixed thoroughly and 0.1 ml. is seeded into the 2 ml. volume of the medium used in the bacteriostatic tests. This inoculum contains between 5,000 and 10,000 organisms.

Bacteriostatic tests performed with cultures diluted in this fashion gave the same results as were obtained when decimal dilutions of culture were made in the ordinary manner.

If these relatively large inocula (5,000 to 20,000 cells) are used in bacteriostatic tests carried out in plain peptone broth, the differences in the susceptibility of various strains of pneumococci to the sulfonamide drugs, and the differences in the bacteriostatic potency of the different sulfonamide drugs on the same strain of bacterium are often obscured. The combined effect of the inhibitor in the medium and the large inoculum is such that growth of a relatively susceptible strain may take place even in the presence of the highest concentrations of the drug that can be dissolved in the medium. Bacteriostatic tests in peptone broth must therefore be carried out with a very small seeding of bacterial cells so that the bacteriostatic effect of a sulfonamide drug may be detected within the limits of its solubility. However, the use of a small inoculum is unsatisfactory for several reasons. In the first place, a very small inoculum of a freshly isolated strain of pneumococcus will often not grow in ordinary artificial medium until the strain has been repeatedly subcultured in this medium. During this process of adaptation some of the characteristics of the freshly isolated strain are obviously modified, and perhaps among others its susceptibility to the sulfonamide drugs. It seems desirable, when testing bacteria for their sulfonamide susceptibility, that the tests be carried out as soon as possible after isolation of the organism. If a larger inoculum is used freshly isolated strains may be tested without requiring a preliminary period of adaptation to artificial media.

Little is known at present concerning the phenomenon of drug resistance in different strains of bacteria. It is possible that the development of drug fastness is the result of a gradual change which takes place in the individual bacterial cells as a result of exposure to the drug. On the other hand drug-fastness may be a manifestation of natural selection, any culture being a variable mixture of drug-susceptible and drug-resistant cells. Until these matters are better understood it seems important that studies of the relative sulfonamide susceptibility of bacterial strains be made upon an adequate sample of the population.

THE MEDIUM

One of the chief sources of error is due to the presence of inhibitors of the sulfonamide compounds in the common bacteriological culture media. MacLeod (1940) has described a satisfactory technique for detecting the presence of sulfonamide inhibitor by the use of a smooth strain of *E. coli* grown in a synthetic medium. Lockwood (1938, 1940) has shown that peptone is inhibitory to the bacteriostatic action of sulfanilamide. More recently it has been demonstrated that different peptones and even different lots of the same brand of peptone contain varying amounts of sulfonamide inhibitor. For this reason it is often difficult to obtain reproducible results in bacteriostatic tests performed with different lots of the ordinary media containing peptone. Moreover, another important ingredient of culture media, namely meat infusion as customarily prepared, also contains sulfonamide inhibitor. It is obvious, therefore, that to

obtain inhibitor-free media the use of these components must be avoided, at least in the form ordinarily employed. For these reasons it seemed important to develop a medium capable of supporting the maximal growth of the more fastidious microorganisms which, at the same time, would be as free as possible of substances known to inhibit the bacteriostatic effect of sulfonamide drugs.

For this purpose a method was devised for preparing extracts of animal organs under conditions yielding the essential nutritive and growth-accessory factors without releasing the drug-inhibitory substances which are liberated or formed during the processes of tissue autolysis. It was found that an infusion of fresh calf or beef liver may be so prepared as to fulfill these requirements and to provide a medium which supports the rapid and profuse growth of pneumococci without the addition of peptone. The preparation of the liver infusion has been described elsewhere in detail (MacLeod, 1940).

Fresh beef or calf liver is obtained at the slaughter house as soon as possible after the death of the animal. The liver is immediately plunged into boiling water and kept at this temperature for 5 to 10 minutes in order to destroy the autolytic ferments. After mincing in a meat chopper the tissue is suspended in twice its weight of tap water, including that used in boiling the organ originally. The pH is adjusted to 4.5 with N/1 HCl, and the infusion heated slowly to 80°C. over a boiling water bath. The liver pulp is then removed by filtration through paper, the pH adjusted to 7.8 with N/1 NaOH, filtered again through fine-grained paper and the fluid then sterilized by filtration through Pasteur-Chamberland candles, bougie B. Filtration is facilitated by warming the infusion to 35°–40°C.

When prepared from calf liver the infusion is clear, golden-yellow in color and strongly fluorescent. Similar infusions made from beef liver have tended to be opalescent, due apparently to the presence of lipids. A seeding of pneumococci or Group A hemolytic streptococci containing only 5 to 10 viable cells grows readily when inoculated into 2 to 5 ml. of infusion. If stored in the dark and in the cold with only a small surface exposed to air, the infusion retains its growth-promoting qualities for a considerable period of time. However, if exposed to light at room temperature, the infusion loses much of its fluorescence within 48 hours with a concomitant loss of its ability to support the growth of small inocula of pneumococci. The growth-promoting qualities of the infusion may be restored completely by the addition of a reducing agent such as sodium thioglycollate (0.1 mgm. per ml.), although the fluorescence does not return. The addition of the sodium thioglycollate at the time the infusion is prepared tends to stabilize the medium and to prevent the formation of deleterious products of oxidation.

Occasional strains of penumococcus have been encountered which do not grow readily in the infusion unless serum is added. To avoid this difficulty, fresh normal rabbit serum in final concentration of 2 per cent has been added routinely. This serum is free of sulfonamide inhibitor, and in addition serves to stabilize the infusion.

In the bacteriostatic tests a volume of 2.0 ml. of medium has been used

throughout. The various concentrations of sulfapyridine have been obtained by making appropriate dilutions in the liver infusion of a 1:2,000 stock saline solution sterilized by heating in a boiling water bath for 5 to 10 minutes.

The content of reducing sugars in different lots of liver infusion has varied between 0.4 and 0.5 per cent. Because of the high carbohydrate content the pH of the medium reaches the acid death point for pneumococcus shortly after maximum growth has taken place. This fact does not interfere with reading the end point in the bacteriostatic tests, and the inhibitor-free liver infusion has been found to be entirely satisfactory for performing these tests. The high sugar content and subsequent acid production in the liver infusion do, however, make this medium unsuitable for storing cultures of pneumococci and for growing the culture to be seeded in the bacteriostatic tests.

REMOVAL OF SULFONAMIDE INHIBITOR FROM PEPTONE BROTH

As previously mentioned, the common bacteriological media prepared from muscle infusion and containing peptone, are unsuitable for bacteriostatic tests because of their content of inhibitor. Media of this sort, however, are desirable for the storage of cultures and for growing cultures to be used for the inoculation of the liver infusion, since the low sugar content does not permit the production during growth of sufficient acid to cause rapid death of the organisms. However, if peptone broth culture is used for inoculation, sufficient sulfonamide inhibitor may be carried over into the liver infusion to affect the outcome of the bacteriostatic tests, even though the serial dilutions are made in the inhibitor-free liver infusion. Accordingly, methods were sought for removing the inhibitor from peptone broth by the use of a selective adsorbent.

The reaction of plain broth prepared with Pfanstiehl's peptone is adjusted to between pH 5.0 and 5.5 with HCl, and for each 100 ml. of medium, 2 grams of powdered charcoal are added. The mixture is then brought to the boiling point over an open flame, and immediately filtered through paper. The reaction is readjusted with NaOH so that the final pH will be 7.8 after sterilization in the Arnold.

The charcoal-adsorbed broth is considerably lighter in color than the original unabsorbed medium, and most of the sulfonamide inhibitor is removed. Moreover, the ability of the adsorbed medium to promote the growth of small inocula of pneumococci is at the same time greatly enhanced. An inoculum of 5 to 50 cells in 5.0 ml. of medium usually grows readily, whereas before adsorption an inoculum of from 1 to 10 million cells is frequently necessary to initiate growth.

In a study of the oxidation-reduction potentials of bacteriological media Dubos (1929 a and b) has shown that peptone broth contains certain substances which become oxidised upon exposure to air, and so alter the oxidation-reduction potential of the medium that the growth of bacteria occurs only when a large inoculum is used. When a medium which has become oxidized by exposure to air is subsequently reduced either by boiling or by the addition of cysteine or thioglycollic acid, the growth-promoting properties are greatly improved. However, when again exposed to air, the autoxidizable substances become

re-oxidized quickly and interfere with the growth of small inocula. In a further investigation of this problem Dubos (1930) showed that various brands of commercial peptone contain different amounts of oxidizable substances which exert a bacteriostatic effect on certain microorganisms. The addition of reducing agents annuls this effect. The bacteriostatic substances may be removed from commercial peptone by acid precipitation, and media prepared with the purified material are capable of supporting the growth of very small inocula of pneumococci.

The improvement in the growth-promoting properties of peptone broth after adsorption with charcoal is much greater than that which occurs when broth is merely heated for the same period in the absence of charcoal. Moreover, even if adsorption is carried out in the cold, the growth-promoting qualities of the medium are greatly improved although not so much as when adsorption is carried out in hot solution.

In order to compare the effect of heat and charcoal adsorption upon the growth-promoting properties of a lot of plain broth prepared with Pfanstiehl's peptone, the reaction was adjusted to pH 5.2 with N/1 HCl, and various portions of the broth treated as described below.

Portion 1. 2 grams of charcoal were added for each 100 ml. of broth. The mixture was brought to a boil over an open flame and immediately filtered through paper. After cooling, the reaction was adjusted to pH 7.8 and the adsorbed broth sterilized by heating in a boiling water bath for 20 minutes.

Portion 2. Instead of carrying out adsorption in hot solution, this procedure was done at room temperature, contact with charcoal being maintained for the same length of time as in the case of Portion 1. After filtration through paper the reaction was adjusted to pH 7.8 and the broth sterilized by filtration through a Berkefeld "V" candle.

Portion 3. In this instance no charcoal was added, but the broth was otherwise subjected to the same procedures as in the case of Portion 1.

The ability of each treated portion and of the original untreated broth to support the growth of varying inocula of Pneumococcus Type I was tested. The results of these tests are shown in table 1.

From table 1 it can be seen that adsorption of plain peptone broth with charcoal in hot solution improves greatly its capacity to promote the growth of small inocula of pneumococci. In the original untreated broth a 10^{-2} ml. inoculum was necessary to inititate growth whereas after adsorption the medium supported the growth of an inoculum of 10^{-8} ml. The difference between these portions of the same lot of broth cannot be explained on the basis of the heating to which the adsorbed broth was subjected, since heat alone in the absence of charcoal improved the growth-promoting properties of the broth only moderately. Moreover, when the broth was adsorbed with charcoal at room temperature and sterilized by filtration, its nutritive properties were greatly improved, indicating that the bacteriostatic substance present in broth is adsorbed by charcoal at pH 5.2 and that the improvement in the broth is not due solely to the heating used in the adsorption procedure. The superiority of broth adsorbed in hot solution to that

adsorbed at room temperature is probably due to more efficient adsorption at the higher temperature. It is worthy of note that charcoal-adsorbed broth even when exposed to air retains its growth-promoting properties for a prolonged period and becomes oxidized very slowly, if at all.

It should be emphasized that adsorption must be carried out between pH 5.0 and 6.0 in order to remove at the same time both the growth-retarding and sulfonamide-inhibiting substances. Below pH 5.0 adsorption removes from the medium certain of its growth-promoting properties, and above pH 6.0 removal of the sulfonamide inhibitor is much less complete than at more acid reaction.

Although the sulfonamide inhibitor and the growth-inhibitory substances are removed from broth by the same procedure, it is not inferred that the two substances are of the same nature. The evidence at present indicates that these two principles are distinct. For example, most of the growth-retarding effect of oxidized broth can be annulled by the addition of a reducing agent such as sodium thioglycollate, whereas the sulfonamide inhibitor is not affected.

TABLE 1

Effect of the growth-promoting properties of charcoal-adsorbed broth and heated broth

MEDIUM	GROWTH OF PNEUMOCOCCUS TYPE I*							
	Inoculum in ml †							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Portion 1 adsorbed with charcoal in hot solution and sterilized by boiling	+	+	+	+	+	+	+	+
Portion 2 adsorbed with charcoal at room temperature and sterilized by filtration	+	+	+	+	+	+	−	−
Portion 3 heated at pH 5.2, readjusted to pH 7.8 and sterilized by boiling. Unadsorbed	+	+	+	+	−	−	−	−
Original untreated broth	+	+	−	−	−	−	−	−

* Readings of growth were made after incubation at 37°C. for 24 hours.

† Dilutions of culture for inoculation were made in plain broth adsorbed with charcoal in hot solution. Inoculum of 10^{-7} ml. contained 80 pneumococci as determined by plate count.

BACTERIOSTATIC TESTS IN DIFFERENT CULTURE MEDIA

Comparative bacteriostatic tests with Pneumococcus Type I were carried out in peptone broth, in peptone broth adsorbed with charcoal in hot solution, and in the liver infusion. The differences in the degree of bacteriostasis caused by sulfapyridine added to the various media are shown in table 2. Inasmuch as normal rabbit serum in final concentration of 2 per cent has been added routinely to the liver infusion, in the present experiments the same amount of serum was added to charcoal-adsorbed and unadsorbed peptone broth. This amount of

serum improves greatly the growth-promoting properties of the peptone broth, and permits the use of small inocula. The normal rabbit serum was shown to be free of sulfonamide inhibitor by *E. coli* test in a synthetic medium, as previously reported (MacLeod, 1940).

The degree of growth was estimated by comparing the gross turbidity of the tubes after incubation at 37°C. for 24 hours. This was found to be a satisfactory growth period, since even with more prolonged incubation the results are almost invariably the same as at 24 hours.

As shown in table 2 the bacteriostatic effect of sulfapyridine varies greatly in different culture media. In liver infusion bacteriostasis was complete in dilutions of sulfapyridine as high as 1:200,000 whereas the same degree of bacteriostasis in unadsorbed peptone broth requires a concentration of 1:5,000. However, after charcoal-adsorption bacteriostasis requires concentrations of the drug considerably less than in the untreated broth indicating that adsorption has removed most

TABLE 2

Bacteriostatic effect of sulfapyridine on pneumococcus type I in different culture media

MEDIUM*	GROWTH OF PNEUMOCOCCUS TYPE I†							
	Concentration of sulfapyridine							
	M/1,250 1:5,000	M/2,500 1:10,000	M/5,000 1:20,000	M/12,500 1:50,000	M/50,000 1:200,000	M/125,000 1:500,000	M/250,000 1·1,000,000	0
Peptone broth.. ...	−	++	+++	++++	++++	++++	++++	++++
Charcoal-adsorbed peptone broth .	−	−	−	+	++++	++++	++++	++++
Liver infusion......	−	−	−	−	−	−	++	++++

* 2 per cent of normal rabbit serum added to each of the various media.

† ++++ = maximum growth; + = slight growth; − = no growth.

Inoculum: 4,000 cells.

The final readings were made after incubation at 37°C. for 24 hours.

of the sulfonamide inhibitor. The concentration of sulfapyridine required for bacteriostasis in the charcoal-adsorbed broth is only moderately greater than that necessary in the liver infusion.

It is important, however, that the medium used for bacteriostatic tests be completely free from sulfonamide inhibitor, since only under these conditions can the results obtained with different drugs or different bacterial strains be compared. As previously shown (MacLeod, 1940) the content of sulfonamide inhibitor varies in different brands of commercial peptones and in infusions of different organs. Complete freedom from inhibitor is most important in the tubes containing higher dilutions of the sulfonamide drugs, since a very small amount of inhibitor will entirely annul the effect of small concentrations of the drug, although in the presence of high concentrations of drug the effect of a small amount of inhibitor may not be so apparent. No lot of liver infusion has been used unless shown by previous testing with the *E. coli* technique to be free of sulfonamide inhibitor.

COMPARISON OF BACTERIOSTATIC TESTS WITH VARIOUS SULFONAMIDE DERIVATIVES IN THE PRESENCE AND ABSENCE OF DRUG INHIBITOR

The differences in the bacteriostatic potency of various sulfonamide drugs may be almost completely masked by the presence of sulfonamide inhibitor in the medium in which bacteriostatic tests are made. This effect is shown in table 3.

It can readily be seen that the striking differences in the bacteriostatic effect of equimolar concentrations of sulfanilamide, sulfadiazine, sulfapyridine, and sulfathiazol on a strain of Type I Pneumococcus, are almost completely masked when the test is performed in ordinary culture medium. These actual differences in the bacteriostatic potency of the four drugs are sharply defined however when inhibitor-free liver infusion is used.

TABLE 3

Effect of the presence of drug inhibitor in media upon the bacteriostatic action of various sulfonamide derivatives

MEDIUM	SULFONAMIDE DRUG	GROWTH OF PNEUMOCOCCUS TYPE I*							
		Concentration of drug							
		M/1000	M/2000	M/4000	M/8000	M/16,000	M/32,000	M/64,000	0
Peptone broth + 2 per cent rabbit serum	Sulfanilamide	++++	++++	++++	++++	++++	++++	++++	++++
	Sulfadiazine	+	++++	++++	++++	++++	++++	++++	++++
	Sulfapyridine	–	+++	++++	++++	++++	++++	++++	++++
	Sulfathiazole	–	++	++++	++++	++++	++++	++++	++++
Liver infusion + 2 per cent rabbit serum	Sulfanilamide	–	+++	++++	++++	++++	++++	++++	++++
	Sulfadiazine	–	–	–	++	++++	++++	++++	++++
	Sulfapyridine	–	–	–	–	–	++++	++++	++++
	Sulfathiazole	–	–	–	–	–	–	++++	++++

* ++++ = maximum growth; + = slight growth

Inoculum: 7,600 cells.

Readings of growth were made after incubation at 37°C for 24 hours

SUMMARY

A technique has been described for estimating the bacteriostatic effect of sulfonamide drugs upon pneumococci. By this method certain of the variables which affect the outcome of these tests may be avoided. It has been shown that the most important source of error in bacteriostatic tests is due to the presence of sulfonamide inhibitor in the usual bacteriological media. This is particularly true in that different lots of the same peptone contain different amounts of sulfonamide inhibitor.

The question of the fluid used in diluting the culture to obtain a suitable inoculum is discussed briefly. In the present study liver infusion has been used since it is not only free from sulfonamide inhibitor and growth-inhibiting substances, but also causes no damage to the bacterial cells.

In carrying out the test a relatively large inoculum of 5,000 to 20,000 cells has been used. By this means a more representative sample of the bacterial popula-

tion is taken, and furthermore if a large inoculum is used strains of pneumococcus may be tested immediately after isolation from the patient, and without the necessity of prolonged adaptation to artificial media. The use of cultures of a standard age in performing bacteriostatic tests with sulfonamides is discussed. This is important since it has been shown by various investigators that older cells are in general less susceptible than young cells to the action of many deleterious physical and chemical agents.

The preparation and use of an inhibitor-free liver infusion are described. This infusion will support the luxuriant growth of various fastidious microorganisms without the addition of peptone, and has been found satisfactory as a culture medium for the performance of the bacteriostatic tests.

A technique is described whereby most of the sulfonamide inhibitor may be removed from plain peptone broth by boiling it with charcoal at an acid reaction. The growth-promoting properties of the broth are also greatly improved by this process due to the simultaneous removal of substances which inhibit growth. Charcoal-adsorbed peptone broth has been found satisfactory for growing the cultures to be used as inocula in the bacteriostatic tests.

By employing these various modifications of technique, readily reproducible results may be obtained in determining the susceptibility of freshly-isolated strains of pneumococci to the action of sulfonamides. Clear cut differences in the potency of various sulfonamide derivatives may also be demonstrated.

BIBLIOGRAPHY

Bayne-Jones, S., and Sandholzer, L. A. 1933 Changes in shape and size of *B. coli* and *B. megatherium* under influence of bacteriophage-motion photomicrographic analysis of mechanism of lysis. J. Exptl. Med., **57**, 279–303.

Colebrook, L., Buttle, G. A. H., and O'Meara, R. A. Q. 1936 Mode of action of p-aminobenzenesulphonamide and prontosil in haemolytic streptococcal infections. Lancet, **2**, 1323–1326.

Domagk, G. 1937 Untersuchungen über den Wirkungsmechanismus des Prontosil bei der experimentellen Streptokokken infektion. Z. klin. Med., **132**, 775–801.

Dubos, R. J. 1929 Observations on oxidation-reduction properties of sterile bacteriological media. J. Exptl. Med., **49**, 507–523.

Dubos, R. J. 1929 Initiation of growth of certain facultative anaerobes as related to oxidation-reduction processes in the medium. Ibid., **49**, 559–573.

Dubos, R. J. 1930 Bacteriostatic action of certain components of commercial peptones as affected by conditions of oxidation and reduction. J. Exptl. Med., **52**, 331–345.

Finkelstone-Sayliss, H., Paine, C. G., and Patrick, L. B. 1937 Bacteriostatic action of p-aminobenzenesulphonamide upon haemolytic streptococci. Lancet, **2**, 792–795.

Fleming, A. 1940 Observations on bacteriostatic action of sulphanilamide and M and B 693 and on influence thereon of bacteria and peptone. J. Path. Bact., **50**, 69–81.

Gates, F. L. 1929 Study of bactericidal action of ultra violet light; reaction to nonchromatic radiations. J. Gen. Physiol., **13**, 231–248.

Green, H. N. 1940 Mode of action of sulphanilamide, with special reference to bacterial growth-stimulating factor ("P" factor) obtained from Br. abortus and other bacteria. Brit. J. Exptl. Path., **21**, 38–64.

Hershey, A. D. 1939 Factors limiting bacterial growth; age of parent culture and rate of growth of transplants of Escherichia coli. J. Bact., **37**, 285–299.

Lockwood, J. S. 1938 Studies on mechanism of action of sulfanilamide; effect of sulfanilamide in serum and blood on hemolytic streptococci *in vitro*. J. Immunol., **35**, 155–193.

LOCKWOOD, J. S., AND LUNCH, H. M. 1940 Studies on mechanism of action of sulfanilamide; influence of proteolytic products on effectiveness of sulfanilamide. J. Am. Med. Assoc., **114**, 935–940.

LONG, P. H., AND BLISS, E. 1939 Clinical Use of Sulfanilamide and Sulfapyridine and Allied Compounds. Macmillan and Co., New York, p. 96–110.

MACLEOD, C. M. 1940 Inhibition of bacteriostatic action of sulfonamide drugs by substances of animal and bacterial origin. J. Exptl. Med., **72**, 217–232.

MACLEOD, C. M., AND MIRICK, G. S. 1941 Bacteriological diagnosis of pneumonia in relation to chemotherapy. Am. J. Pub. Health, **31**, 34–38.

NITTI, F., BOVET, D., AND DEPIERRE, F. 1937 Action des dérivés du p-aminophénylsulfamide (1162F) sur les streptocoques hemolytiques in vitro. Compt. rend. soc. biol., **124**, 16–18.

ROSENTHAL, S. M. 1937 Studies in chemotherapy; effect of p-aminobenzene sulphonamide on pneumococci in vitro. U. S. Pub. Health Repts., **52**, 192–196.

SCHULTZ, J. A., AND RITZ, H. 1910 Die Thermoresistenz junger und alter Coli-Bacillen. Zentr. Bakt. Parasitenk., **I**, Orig., **54**, 283–288.

SHERMAN, J. M., AND ALBUS, W. R. 1923 Physiological youth in bacteria. J. Bact., **8**, 127–139.

STAMP, T. C. 1939 Bacteriostatic action of sulphanilamide in vitro; influence of fractions isolated from haemolytic streptococci. Lancet, **2**, 10–17.

WINSLOW, C.-E., A., AND WALKER, H. H. 1939 Earlier phases of bacterial culture cycle. Bact. Revs., **3**, 147–186.

WOODS, D. D., AND FILDES, P. 1940 The anti-sulphanilamide activity of p-aminobenzoic acid and related compounds. Chem. Ind., **59**, 133–134.

A DESCRIPTION OF AN UNUSUAL SALMONELLA TYPE WITH SPECIAL REFERENCE TO THE EVOLUTION OF SALMONELLA SPECIES[1]

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The bacillus described here was isolated by Dr. Henry Welch from rat feces collected in the vicinity of Salinas, California. The organism, which is designated as *Salmonella salinatis*, was identified by Dr. Welch as a member of the genus *Salmonella* and sent to the writers for further study. On examination it proved to be a motile rod which possessed the morphological, tinctorial, and biochemical characters of *Salmonella*. The bacillus produced hydrogen sulfide, did not form indole, and failed to liquefy gelatin. Glucose, arabinose, maltose, rhamnose, trehalose, xylose, sorbitol and dulcitol were fermented promptly with the production of acid and gas. Lactose, sucrose, inositol, adonitol, and salicin were not attacked. Dextro-tartrate, levo-tartrate, mucate and citrate were utilized by the bacillus but utilization of meso-tartrate could not be demonstrated.

Serological examination of the organism revealed that it was a member of group B of the Kauffmann-White classification and possessed the somatic antigens *IV*, *XII*.... An agglutinating serum was prepared for *S. salinatis* and it was found that all O agglutinins were removed from the serum by absorption with the Sandiego type (IV, XII .: $e, h - e, n, z_{15}$).

The determination of the flagellar antigens of the bacillus presented greater difficulties. Broth cultures were sluggishly motile. When diluted with formalized saline and tested with serums representing the various H factors of the Kauffmann-White schema, the broth cultures were flocculated slowly by serum derived from *Eberthella typhosa* (d) and still more slowly by serum derived from the beta phase of the Mikawashima type (e, n, z_{15}). The organism was passed through semi-solid agar until a very motile culture was obtained. This culture was flocculated rapidly and to the titre of *E. typhosa* serum as well as all other serums containing agglutinins for the antigen d. It was also actively flocculated by all serums containing agglutinins for antigens e, n and to a lesser extent by e, h serums. This suggested that the organism was diphasic and possessed the H formula $d - e, n$...which is known to exist in the Amersfoort type. Such a culture on plating should give rise to colonies some of which contain antigen d while others contain antigen e, n.... This was not true. The culture was plated on a number of occasions and several hundred individual colonies examined. Each colony examined contained antigen d as the major H

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

constituent. Some of the colonies also were agglutinated actively by *e*, *n*... serums and to a lesser degree by *e*, *h* serums. When tested with single factor *h* and *n* serums, colonies of this sort were flocculated by *n* serum but not by *h* serum. Other colonies when tested on a slide, or when planted in broth and used as antigens in tube tests, reacted only with *d* serum. If the latter colonies were picked to semi-solid agar and then transferred to broth to insure very active motility, it was found that the organisms were flocculated not only by *d* serum but that they also reacted in low dilutions of *e*, *h* and *e*, *n*...serums. When tested with *h* and *n* serums, these colonies flocculated slowly and slightly in *h* serum but were unaffected by *n* serum. The agglutinative reactions of the isolated colonies and of the cultures used for comparison are given in table 1.

TABLE 1

Agglutination reactions of phases of S. salinatis

ANTIGENS	SERUMS						
	S. salinatis, phase 1	*S. salinatis*, phase 2	*E. typhosa*	*S. anatum*, phase 1	*Mikawashima*, phase 2	*h*	*n*
S. salinatis, phase 1	20,000	20,000	20,000	2,000	1,000	+	—
S. salinatis, phase 2	20,000	20,000	20,000	1,000	10,000	—	+
E. typhosa (*d*)	20,000	20,000	20,000	—	—	—	—
S. anatum, phase 1 (*e*, *h*)	500	1,000	—	40,000	500	+	—
Mikawashima, phase 2 (*e*, *n*, z_{15})	500	5,000	—	500	20,000	—	+

Figures indicate highest dilution at which agglutination occurred.
— indicates absence of agglutination at 1–100.
+ indicates agglutination in *h* or *n* serum at 1–500.

It is evident that two antigenically different phases are present in the culture and that these can be separated by colony selection. In both phases *d* is the dominant antigen and this dominance tends to mask their differential characters. This is particularly true of phase 1, in which the minor components are present only in very slight amount. The results indicate that the antigens *d*, *e*, *h* and *n*... are present and that phase 1 carries *d*, *e*, *h* while phase 2 contains *d*, *e*, *n*.... This supposition is confirmed by the absorption tests given in table 2. Serum derived from phase 1 of *S. salinatis*, after absorption with *Salmonella anatum*, phase 1 (*e*, *h*), still agglutinates phases 1 and 2 of *S. salinatis* and *E. typhosa* strongly because of the high *d* titre of the serum. However it no longer agglutinates *e*, *h* or *e*, *n*... forms. If the serum is absorbed with phase 2 of the Mikawashima type (*e*, *n*, z_{15}) it still retains its *d* content and agglutinates *S. salinatis* and *E. typhosa*. In addition there is a slight residue of agglutinins left for *e*, *h* phases but none for *e*, *n*... phases. This demonstrates that in addition to the dominant antigen *d*, phase 1 contains very small amounts of *e*, *h* and that the phase may be expressed as *d*, *e*, *h*. The *e*, *h* component of this phase is so poorly developed that when serum derived from phase 1 of *S. anatum* (*e*, *h*) is absorbed with phase 1 of *S. salinatis* a pronounced residue of *e*, *h* agglutinins

remains even though very large absorbing doses are used. This might be interpreted as indicating that the *e*, *h* antigens of phase 1 were not typical and that failure to absorb was due to a qualitative rather than a quantitative difference. It is believed that this interpretation is not correct since the results given in the second part of the paper demonstrate that a perfectly typical *e*, *h* phase can be obtained from phase 1 of *S. salinatis*. The lack of absorbing power apparently is due only to the slight amount of *e*, *h* antigens in phase 1.

If serum derived from phase 2 of *S. salinatis* is absorbed by phase 1 of *S. anatum* the dominant *d* agglutinin for *S. salinatis* and *E. typhosa* remains. All agglutinins for the *e*, *h* phases are removed but a well-defined residue of agglutinins for the *e*, *n*... phases remains. If the serum is absorbed with *Salmonella*

TABLE 2

Agglutinin absorption tests with phases of S. salinatis

ANTIGENS	SERUMS: *S. salinatis*, phase 1 absorbed by *S. anatum*, phase 1	SERUMS: *S. salinatis*, phase 1 absorbed by Mikawashima, phase 2	SERUMS: *S. salinatis*, phase 2 absorbed by *S. anatum*, phase 1	SERUMS: *S. salinatis*, phase 2 absorbed by *S. abortus-equi*, phase 2	SERUMS: *S. salinatis*, phase 2 absorbed by Mikawashima, phase 2	Mikawashima, phase 2 absorbed by *S. salinatis*, phase 2	*S. anatum*, phase 1 absorbed by *S. salinatis*, phase 1
S. salinatis, phase 1 (*d*, *e*, *h*)	20,000	20,000	10,000	10,000	10,000	—	—
S. salinatis, phase 2 (*d*, *e*, *n*, z_{15})	10,000	20,000	20,000	10,000	10,000	—	100
E. typhosa (d)	10,000	10,000	10,000	10,000	10,000	—	—
S. anatum, phase 1 (*e*, *h*)	—	200	—	—	—	—	5000
Newport, phase 1 (*e*, *h*)	—	200	—	—	—	—	5000
S. abortus-equi, phase 2 (*e*, *n*, *x*)	—	—	2,000	—	—	—	200
Mikawashima, phase 2 (*e*, *n*, z_{15})	—	—	2,000	200	—	—	200

Figures indicate highest dilution at which agglutination occurred.
— indicates absence of agglutination at 1–100.

abortus-equi (*e*, *n*, *x*), agglutinins for the *e*, *h* phases and for the absorbing strain are removed but a small amount of agglutinin acting on phase 2 of Mikawashima (*e*, *n*, z_{15}) remains. This indicates that phase 2 of *S. salinatis* contains the antigens *e*, *n*, z_{15}. This view is strengthened by the results of the absorption of the serum with phase 2 of Mikawashima, which leaves only the agglutinins for antigen *d* in the serum. Finally, absorption of serum derived from phase 2 of Mikawashima by phase 2 of *S. salinatis* removes all agglutinins for the homologous strain. It may be concluded that phase 2 of *S. salinatis* contains the antigens *d*, *e*, *n*, z_{15}.

All the phases of the genus *Salmonella* which contain antigen *d* cross agglutinate in very high dilution, usually to the titres of the serums. In spite of this close relationship of antigen *d* contained in different types, minimal differences

in these *d* phases can be demonstrated by absorption. These differences have been commented upon by Kauffman (1937), by Hormaeche and Peluffo (1939) and by Edwards and Bruner (1941). In order to determine more exactly the relationships of the *d* component of *S. salinatis* to that of other types cross absorption tests were done. The results of these absorptions are given in table 3. The *S. salinatis* serum used in the tests was derived from phase 1 and was subjected to a previous absorption with the Sandiego type to remove the small amount of *e, h* agglutinins which it contained. Absorption of *S. salinatis* serum with the Shangani, Gaminara, or Oregon types removed all H agglutinins for the homologous strain. Absorption with *E. typhosa,* Muenchen or Stanley left residues of agglutinins in the serum. *S. salinatis* did not completely remove agglutinins from *E. typhosa,* Muenchen, or Oregon serums. The results indicate

TABLE 3

Serological relationships of antigen d of S. salinatis

ANTIGENS	SERUMS								
	S. salinatis absorbed by						*E. typhosa* absorbed by *S. salinatis*	Muenchen absorbed by *S. salinatis*	Oregon absorbed by *S. salinatis*
	Shangani	Oregon	Gamanara	Muenchen	Stanley	*E typhosa*			
S. salinatis, phase 1	−	−	−	+	+	+	−	−	−
E. typhosa	−	−	−	−	−	−	+	+	+
Gaminara, phase 1	−	−	−	+	−	+	−	+	+
Stanley, phase 1	−	−	−	−	−	+	+	+	+
Muenchen, phase 1	−	−	−	−	−	−	+	+	+
Shangani, phase 1	−	−	−	+	−	+	+	+	+
Wichita	−	−	−	−	−	+	−	−	+
Oregon, phase 1	−	−	−	+	−	+	−	+	+
Amersfoort, phase 1	−	−	−	+	−	+	+	+	+

+ agglutination at 1–100.
− no agglutination at 1–100.

that the antigen *d* of *S. salinatis* is very closely related to that of Oregon, Gaminari, and Shangani but that it lacks some of the minor components of those types.

Twelve transplants of *S. salinatis*, each of which was isolated from a single colony, were maintained on agar slants and transferred at weekly intervals. When isolated, six of these subcultures were phase 1, the other six were phase 2. After three transfers, each of the subcultures was plated and the antigens of individual colonies were determined. It was found that the six subcultures originally isolated from phase 1 colonies gave rise to colonies, the majority of which were phase 1. However, some phase 2 colonies were found in each of the subcultures. The number of phase 2 colonies in the different subcultures varied from 5 per cent to 30 per cent of the colonies examined. The subcultures isolated from phase 2 colonies all gave rise to some phase 1 colonies when they were

plated. As in the case of the phase 1 subcultures, the percentage of colonies which reverted to the other phase was variable. These results demonstrate that the culture is diphasic and exhibits natural phase variation.

It may be concluded that *S. salinatis* is a diphasic *Salmonella* and that it is represented by the antigenic formula $IV, XII \ldots : d, e, h - d, e, n, z_{15}$.

INDUCED VARIATION IN SALMONELLA SALINATIS

It should be emphasized that the foregoing results were obtained with naturally occurring phases of the bacillus and that in no instance were the organisms cultivated in immune serums in order to induce variation. The writers (Bruner and Edwards, 1939, 1941; Edwards and Bruner, 1939, 1940) have demonstrated that suppressed phases of the monophasic nonspecific types, of *Salmonella paratyphi A* and of *S. abortus-equi* could be made apparent by the cultivation of the bacilli in the presence of serums containing agglutinins for their dominant phases. The results obtained in these studies supported the view of White (1926) that current *Salmonella* types were loss variants of antigenically complex parent strains. Since the antigenic composition of *S. salinatis* was more complex than that of any previously described *Salmonella* type it seemed an excellent strain with which to attempt the production of loss variation.

In attempting to produce variation a modification of the method of Gard (1937) was used. This procedure was described by Bruner and Edwards (1939) and is based on the immobilizing effect of agglutinating serum upon motile organisms. Semi-solid agar containing agglutinating serum for the organism under study is inoculated by stabbing. The resulting growth is confined to the line of stab unless changes occur in the flagellar antigens, in which case the bacilli migrate through the medium. The organism was first cultivated in semi-solid agar containing $e, h; e, n; h; n; e, h + e, n;$ and $h + n$ serums, respectively. The serums were used in a dilution of one fifth of the agglutinin titre, i.e. a serum having a titre of 1 to 20,000 was used in a dilution of 1–4000. When these serums were used singly or in combination the organisms migrated through the medium. The results obtained with phases 1 and 2 in semi-solid agar containing $e, h; e, n, z_{15}$ and $e, h + e, n, z_{15}$ serums are given in table 4. The results obtained with absorbed single factor h and n serums were the same. When phase 1 (d, e, h) was cultivated in semi-solid agar with an e, h serum, both phase 1 and phase 2 were recovered from the spreading growth. When phase 1 was cultivated in e, n, z_{15} serum only phase 1 migrated through the medium. If both e, h and $e, n \ldots$ serums were added to the medium, phase 1 still spread through the agar. The amounts of serum were increased tenfold and phase 1 still spread through the medium. The *S. anatum* serum, which had a titre of 1–40,000 was then added to the medium in amount of 1 ml. to 3 ml. of semi-solid agar while the e, n, z_{15} serum was added in the amount previously used. Even in this high concentration of serum, phase 1 continued to spread. The small amount of the e, h components in phase 1 was commented upon above. Apparently these antigens are present in such slight amount that the phase is not immobilized by e, h serums regardless of the concentration in which they are used.

The results obtained by the cultivation of phase 2 in *e, h; e, n,* z_{15} and *e, h* + *e, n,* z_{15} serums were similar to those obtained with phase 1. Phase 2 spread unchanged through semi-solid agar containing *e, h* serum. If agar containing *e, n,* z_{15} serum, or *e, h* + *e, n,* z_{15} serum was inoculated with phase 2 the organisms of that phase were immobilized and phase 1 was recovered from the spreading growth. The addition of *e, h* and *e, n,* z_{15} serums to the medium did nothing more than cause the organism to exhibit normal phase variation. The organisms which had been exposed to these serums, and which migrated through the medium in their presence were apparently unchanged. After their isolation from the tubes containing large amounts of serum they continued to display phase

TABLE 4

Agglutination of S. salinatis after growth in presence of agglutinating serum

ANTIGENS	SERUMS ADDED TO MEDIUM	SERUMS				
		E. typhosa	*S. anatum*, phase 1	Mikawashima, phase 2	*h*	*n*
S. salinatis, phase 1 cultivated in	*S. anatum*, phase 1 (*e, h*)	20,000	1,000	5,000	+	+
	Mikawashima, phase 2 (*e, n,* z_{15})	20,000	1,000	500	+	−
	S. anatum, phase 1 + Mikawashima, phase 2	10,000	1,000	200	+	−
	E. typhosa	−	20,000	500	+	−
	E. typhosa + *h*	−	1,000	20,000	−	+
	E. typhosa + *n*	−	20,000	500	+	−
S. salinatis, phase 2 cultivated in	*S. anatum*, phase 1 (*e, h*)	10,000	500	5,000	−	+
	Mikawashima, phase 2 (*e, n,* z_{15})	20,000	1,000	500	+	−
	S. anatum, phase 1 + Mikawashima, phase 2	10,000	1,000	500	+	−
	E. typhosa	−	1,000	20,000	−	+
	E. typhosa + *h*	−	1,000	20,000	−	+
	E. typhosa + *n*	−	20,000	500	+	−

Figures indicate highest dilution at which agglutination occurred.
− indicates absence of agglutination at 1–100.
+ indicates agglutination in *h* or *n* serum at 1–500.

variation in the absence of serum. The failure to induce variation must be attributed to the failure of *e, h* serum to immobilize the *d, e, h* phase of the bacillus.

Entirely different results were obtained when the organisms were grown in semi-solid agar containing *E. typhosa* serum or any other serum that contained agglutinins for antigen *d*. The results of these experiments are summarized in table 4. If phase 1 of *S. salinatis* (*d, e, h*) is placed in semi-solid agar containing *E. typhosa* serum the organisms spread rapidly through the medium. Cultures isolated from the spreading growth have undergone profound changes in their antigenic characters. They no longer are agglutinated by *E. typhosa* serum but they are agglutinated to the titre of *S. anatum* (*e, h*) serum and to a lesser extent

by Mikawashima (*e*, *n*, z_{15}) serum. When tested with single factor *h* and *n* serums, they are agglutinated by *h* serum but not by *n* serum. They behave as a typical *e*, *h* phase, which occurs naturally in phase 1 of many *Salmonella* types. If, in addition to *E. typhosa* serum, single factor *n* serum is added to the medium phase 1 behaves in the same way. If, in addition to *E. typhosa* serum, single factor *h* serum is added to the medium the organisms spread through the agar but the spreading growth possesses different antigenic characters. It is not agglutinated by *E. typhosa* serum and is agglutinated only in low dilution with *S. anatum* serum. It reacts to the titre of Mikawashima serum and when tested with single factors, reacts with *n* serum but not with *h* serum. It resembles the naturally occurring *e*, *n*... antigens of phase 2 of many Salmonella types.

When the experiments described above were repeated with phase 2 of *S. salinatis* (*d*, *e*, *n*, z_{15}) comparable results were obtained. When cultivated in *E. typhosa* serum, or *E. typhosa* + *h* serum, an *e*, *n*... phase was isolated from the spreading growth. If *E. typhosa* serum + *n* serum were added to the medium an *e*, *h* phase was isolated.

If either phase were planted in semi-solid agar containing *h* and *n* serums in addition to *E. typhosa*, serum, no spreading occurred. Further, either *e*, *h* serum or *e*, *n*... serum, when used in conjunction with *E. typhosa* serum retarded spreading of the organisms. Apparently the agglutinin in the serums for the antigen *e* which is common to both of the phases was sufficiently active to cause this retardation of migration. It was demonstrated previously that when phase 1 (*d*, *e*, *h*) was placed in *e*, *h* serum it was not immobilized and that phase 1 as well as phase 2 could be isolated from the spreading growth. In contrast, when *E. typhosa* serum, as well as *h* serum, is added to the medium the *e*, *h* components no longer spread, as evidenced by the isolation of pure *e*, *n*... phases under these conditions. This difference in action apparently is due to the change in the *e*, *h* antigens from a very minor component of the natural phase 1 to a major component under the influence of *E. typhosa* serum.

After the *e*, *h* and *e*, *n*... phases were isolated they were plated and subcultures were made from isolated colonies. Six colonies of each phase were selected and transferred to agar slants. The subcultures were transferred at weekly intervals and after three weeks they were plated and the colonies were examined. In each of the subcultures which originated from single *e*, *h* colonies, there appeared some *e*, *n*... colonies. The proportion of these *e*, *n*... colonies in the different subcultures varied from 10 per cent to 40 per cent. In each of the subcultures which were isolated from *e*, *n*... colonies some *e*, *h* colonies appeared when they were plated. As in the case of the subcultures which originated from *e*, *h* colonies, the percentage of colonies of the opposite phase varied in different cultures. None of the colonies from *e*, *h* or *e*, *n*... subcultures was agglutinated by *E. typhosa* serum. It would appear that *S. salinatis*, which in its original state exhibited $d, e, h - d, e, n, z_{15}$ phase variation, after passage through *E. typhosa* (*d*) serum had lost antigen *d* and now displayed $e, h - e, n \ldots$ phase variation. The induced *e*, *n*... phase was examined more closely to determine its minor antigens. It was not agglutinated by *x* serum, but flocculated actively with z_{15} serum. It

left a pronounced residue of agglutinins in *S. abortus-equi* (e, n, x) serum but completely removed agglutinins from phase 2 Mikawashima (e, n, z_{15}) serum when used as antigen in absorption tests. The induced phase, therefore, may be expressed as e, n, z_{15}.

Repeated attempts were made to recover the d, e, h − d, e, n, z_{15} form of the original culture from the e, h − e, n, z_{15} form of the variant. These attempts included allowing cultures to age and then plating them and the maintenance of cultures with regular periods of transfer and plating at various intervals. In addition, numerous cultures were planted in semi-solid agar containing e, h and e, n, z_{15} serums or h and n serums. These cultures were transferred many times in semi-solid agar containing these serums. Serial transfer in such mediums is the most effective method known for the isolation of suppressed antigens. These induced e, h − e, n, z_{15} phases have been maintained in the laboratory for a

TABLE 5

Serological characteristics of induced phases of S. salinatis

ANTIGENS	SERUMS			
	S. salinatis e, h phase	*S. salinatis*, e, n, z_{15} phase	Sandiego, phase 1	Sandiego, phase 2
E. typhosa	—	—	—	—
S. salinatis, phase 1	2,000	2,000	1,000	1,000
S. salinatis, phase 2	5,000	5,000	2,000	5,000
S. salinatis; e, h phase	20,000	10,000	5,000	2,000
S. salinatis; e, n, z_{15} phase	10,000	40,000	2,000	5,000
Sandiego, phase 1	20,000	10,000	5,000	2,000
Sandiego, phase 2	5,000	20,000	2,000	5,000

Figures indicate highest dilution at which agglutination occurred.
— indicates absence of agglutination at 1–100.

year and during that time attempts to revert them to the original phase or to recover antigen d from them have been carried on almost constantly. In no instance has any variation from the induced e, h − e, n, z_{15} pattern been observed. Therefore the transformation of the d, e, h − d, e, n, z_{15} phases to the e, h − e, n, z_{15} phases must be considered as an irreversible change.

It should be emphasized that no change occurred in the somatic antigens of the bacillus during the experiments. The antigenic formula of the original culture was IV, $XII\ldots$: d, e, h − d, e, n, z_{15} while that of the variant was IV, $XII\ldots$: e, h − e, n, z_{15}.

Agglutinating serums were prepared from the induced e, h and e, n, z_{15} phases and from phase 1 and phase 2 of the Sandiego type (IV, $XII\ldots$: e, h − e, n, z_{15}) of Kauffmann (1940). The reactions obtained with these serums are given in table 5. No differences are discernible in the action of the serums derived from the induced phases of *S. salinatis* and those derived from the Sandiego type. It is worthy of note that serums derived from the induced phases contained no flocculating agglutinins for *E. typhosa*. This is further evidence of the complete

absence of antigen *d* in the induced phases. When reciprocal absorption tests were performed it was found that the induced phases of *S. salinatis* removed all agglutinins from serums derived from the corresponding phases of the Sandiego type. Likewise the phases of Sandiego removed all agglutinins from the serums derived from the corresponding induced phases of *S. salinatis*. This removal of agglutinins applies to the O antigens as well as the H antigens. Thus the serological identity of the two organisms is proven and the antigenic formula of the induced variant of *S. salinatis* established as $IV, XII\ldots: e, h - e, n, z_{15}$.

DISCUSSION

The results obtained in the study of *S. salinatis* naturally raise the question of the purity of the culture. It should be remembered that the culture was plated many times during the course of the work and, of the hundreds of colonies examined serologically, not one was found which did not possess the antigens attributed to the naturally occurring form of *S. salinatis*. Further, practically all of the work on induced variation was done with cultures isolated from single colonies. More than 100 single colonies were cultivated in semi-solid agar containing *E. typhosa* serum. In all these colonies antigen *d* was the major component. From every one the $e, h - e, n, z_{15}$ form could be obtained. It is inconceivable that in a mixture of two *Salmonella* types, each of the numerous colonies examined should be mixed. The failure to find $e, h - e, n, z_{15}$ forms in the original culture and the isolation of such forms from every colony cultivated in *E. typhosa* serum is proof that a mixture of types was not present in the original culture. The biochemical characteristics of the original culture and the induced forms were identical.

The two phases of *S. salinatis* (d, e, h and d, e, n, z_{15}) are unusually complex. No other *Salmonella* has been described in which similarly complex phases occur. The antigen *d* is found in many *Salmonella* types and in them this antigen alone constitutes a complete phase. It may occur alone in monophasic organisms, as in *E. typhosa* and the Wichita type ($I, XIII, XXIII: d: -$) of Schiff and Strauss (1939), or it may constitute one phase of a diphasic type, as in Muenchen ($VI, VIII: d - 1, 2$) or Amersfoort ($VI, VII: d - e, n, x$). The e, h and e, n, z_{15} antigens each constitutes a complete phase of a number of *Salmonella types*, as in the Sandiego type ($IV, XII\ldots: e, h - e, n, z_{15}$), *S. anatum* ($III, X, XXVI: e, h - 1, 6$) and the Mikawashima type ($VI, VII: y - e, n, z_{15}$).

The fact that *d* is the dominant antigen in both phases of *S. salinatis* makes the recognition of the phases more difficult than in other *Salmonella* types. This difficulty is further complicated by the presence of the common antigen *e* in the minor components of the two phases. The e, h components of the d, e, h phase are present in such small amount, or are so poorly developed, that their recognition is sometimes most difficult. For some time after the separation of the phases the writers were of the opinion that certain colonies contained only antigen *d* and that the bacillus existed in three phases which could be expressed as $d - d, e, h - d, e, n, z_{15}$. Further studies demonstrated that the supposed *d* phase was actually d, e, h. The failure to recognize the e, h components in these

instances was due to lack of maximum development of the H antigens. Certain lots of infusion broth which were being used did not yield cultures which were sufficiently rich in these substances to permit the detection of traces of *e*, *h* antigens. It was noted above that when phase 1 of *S. salinatis* was placed in *E. typhosa* serum, *e*, *h* phases were isolated and that phase 2 under the same conditions yielded *e*, *n*, z_{15} phases. Large numbers of colonies of both phases were examined and only very rarely did a *d*, *e*, *h* phase give rise to an admixture of *e*, *h* and *e*, *n*, z_{15} phases. It was found that the supposed *d* phase, when cultivated in a medium containing *E. typhosa* serum gave rise to an *e*, *h* phase. This occurred in the case of every "*d*" colony so examined. Serum prepared from such a colony contained traces of *e*, *h* agglutinins but not *e*, *n*, z_{15} agglutinins. When the organisms were cultivated in semi-solid agar containing both *e*, *h* and *e*, *n*, z_{15} serums the *d*, *e*, *h* phase was invariably isolated from the spreading growth. This also is evidence against the existence of a pure *d* phase in the culture. The induction of a fully developed, typical *e*, *h* phase from such a poorly developed partial antigen is a curious phenomenon. The *e*, *n*, z_{15} antigens of the *d*, *e*, *n*, z_{15} phase are much more fully developed. One would expect, *a priori*, that the minor components of the two phases would exhibit approximately equal development, particularly since the *e*, *h* and *e*, *n*, z_{15} antigens of the induced phases are equally well developed and typical.

The *e*, *h* – *e*, *n*, z_{15} phases recovered from *S. salinatis* by cultivation in a medium containing *d* serum were surprisingly resistant to further change. Normal phase variation was apparent and the *e*, *h* phase was readily isolated from the *e*, *n*, z_{15} phase by cultivation in a medium containing *n* serum. The reverse change from *e*, *h* to *e*, *n*, z_{15} was also easily accomplished. This variation represented the extent of change in the induced forms. When cultivated in *e*, *h* + *e*, *n*. . . serums or *h* + *n* serums the organisms could not be forced to develop a phase which contained antigen *d*, as did the original culture. Further, no "artificial" phases, i.e. phases having little or no relationship to the naturally occurring H antigens of the genus, were obtained when the organisms were cultivated serially in a medium containing such serums. The production of artificial phases from *E. typhosa* was described by Kauffmann (1936) and from the Schleissheim type by Kauffmann and Tesdal (1937). While the attempts to induce variation in the *e*, *h* – *e*, *n*, z_{15} forms of *S. salinatis* were in progress, two cultures of the Sandiego type were used in parallel studies. Both of these Sandiego cultures spread through semi-solid agar containing *h* + *n* serum after three transfers in the medium. The induced phases thus obtained had no relationships to the natural H antigens of the genus but were related to the artificial phase of the Schleissheim type (z_5). The stability of the *e*, *h* – *e*, *n*, z_{15} forms isolated from *S. salinatis* and the complete absence of *d* agglutinins in serums prepared from them are evidence of the profundity and permanence of the change that has occurred in the culture. The permanence of this change is even more significant when one considers the results obtained in the study of *S. abortus-equi* (Edwards and Bruner, 1939) and *S. paratyphi A* (Bruner and Edwards, 1941). In both of these monophasic types it was possible to induce a

second phase which resembled the naturally occurring antigens of other types. While the induced phases of these species did not revert spontaneously to the naturally occurring phases, they were reverted by cultivation in mediums containing appropriate serums. This is in direct contrast to the irreversibility of the induced phases of *S. salinatis*. The change in the latter species apparently is not a simple variation but is a mutation.

Since the transformation of *S. salinatis* from $d, e, h - d, e, n, z_{15}$ to $e, h - e, n, z_{15}$ is accomplished so easily in the test tube simply by cultivating it in the presence of *d* serum, it is not inconceivable that the same change should take place in the body of an animal whose serum contained agglutinins for antigen *d*. Six guinea pigs which had been injected with killed cultures of phase 1 of the Muenchen type until their *d* agglutinin titre rose to 1 to 1000 were injected with living cultures of *S. salinatis*. The animals withstood large amounts of the culture without apparent ill effect and this question was not pursued further.

In his classical work on the *Salmonella* group, White (1926) discussed the evolution of types at some length. He concluded that in view of the numerous antigenic relationships in the genus different types must have had a common ancestry and that the organisms as they exist today have evolved by loss variation. Through this process of loss certain antigens became dominant in some types, other antigens became dominant in other types. The writers have felt that White was correct in his deductions regarding *Salmonella* phylogeny and have been able to bring forward some evidence to support his theories. Edwards and Bruner (1939) isolated antigen *a* from the naturally monophasic *S. abortus-equi* ($IV, XII \ldots : - : e, n, x$) and Bruner and Edwards (1941) recovered $1, 5 \ldots$ phases from *S. paratyphi A* ($I, II, XII \ldots : a : -$). When the suppressed phases of these two bacilli were isolated they did not spontaneously revert to the original phases. These suppressed phases are isolated with difficulty. Long series of transfers in mediums containing serum usually are necessary to cause their appearance. This indicates that the monophasic state is deeply seated in these two species. The lack of phase variation in the suppressed phases after they are brought into dominance supports this view. These facts indicate that once an organism has lost the power of phase variation, that power is not revived even when suppressed phases are brought into dominance. These induced variants of *S. paratyphi A* and *S. abortus-equi* are *additive* variants and, if the theory of White is accepted, are, in a sense, anachronisms. The organisms have not been forced forward in their evolutionary course, but backward.

Unlike the changes described in *S. abortus-equi* and *S. paratyphi A*, the change induced in *S. salinatis* results in a *loss* of antigenic components. This, according to White's view, represents a step in the natural evolution of the organism. It is not surprising, therefore, that the induced phases of *S. salinatis* should act like those of any other diphasic *Salmonella* and be subject to natural, spontaneous phase variation.

The acceptance of White's theory presupposes the previous existence of an antigenically complex prototype. *S. salinatis* is, of course, not nearly so complex as this prototype must necessarily have been. However, its phases are

more complex than those of any *Salmonella* yet described. It is undoubtedly a more primitive type than any other now known and its behavior indicates that it is in a highly unstable state.

Finally, attention should be called to the fact that in the transformation of *S. salinatis* from $IV, XII \ldots d, e, h - d, e, n, z_{15}$ to $IV, XII \ldots : e, h - e, n, z_{15}$ an organism biochemically and serologically indistinguishable from the Sandiego type has been induced.

CONCLUSIONS

1. A new *Salmonella* type, *Salmonella salinatis*, is described. The antigenic formula for this type is $IV, XII \ldots : d, e, h - d, e, n, z_{15}$.

2. Through cultivation of *S. salinatis* in semi-solid agar containing agglutinating serum for *Eberthella typhosa*, an organism having the antigenic formula $IV, XII \ldots : e, h - e, n, z_{15}$ was isolated. The latter is biochemically and serologically indistinguishable from the Sandiego type.

3. The bearing of these observations, and of previous experiments on induced variation, on White's theory of Salmonella phylogeny is discussed.

REFERENCES

Bruner, D. W., and Edwards, P. R. 1939 A note on the monophasic non-specific *Salmonella* types. J. Bact., **37**, 365–370.

Bruner, D. W., and Edwards, P. R. 1941 The demonstration of nonspecific components in *Salmonella paratyphi A* by induced variation. J. Bact., **42**, 467–478.

Edwards, P. R., and Bruner, D. W. 1939 The demonstration of phase variation in *Salmonella abortus-equi*. J. Bact., **38**, 63–72.

Edwards, P. R., and Bruner, D. W. 1940 The antigenic analysis of Salmonella species derived from domestic animals. Proc. Third Intern. Congr. Microbiol., 629–630.

Edwards, P. R., and Bruner, D. W. 1941 Two new *Salmonella* types closely related to *Salmonella muenchen*. Am. J. Hyg., **34**, 121–124.

Gard, S. 1937 Ein Colistamm mit Salmonella-H-Antigen, zugleich ein Beitrag zur Frage der Definition der Salmonellagruppe. Z. Hyg. Infektionskrankh., **120**, 59–65.

Hormaeche, E., and Peluffo, C. A. 1939 Estudios sobre Salmonella gaminara. Arch. urug. med., cirugia y especialid, **14**, 217–226.

Kauffmann, F. 1936 Über die diphasische Natur der Typhusbacillen. Z. Hyg. Infektionskrankh., **119**, 104–118.

Kauffmann, F. 1937 Salmonella Probleme. Z. Hyg. Infektionskrankh., **120**, 177–197.

Kauffmann, F. 1940 Über den α-β Phasenwechsel in der Salmonellagruppe. Acta Path. Microbiol. Scand., **17**, 430–437.

Kauffmann, F., and Tesdal, M. 1937 Über zwei neue Salmonellatypen mit α-β Phasenwechsel., Z. Hyg. Infektionskrankh., **120**, 168–176.

Schiff, F., and Strauss, L. 1939 A new *Salmonella* type (*Salmonella wichita*) J. Infectious Diseases, **65**, 125–126.

White, P. B. 1926 Further studies of the *Salmonella* group. Med. Research Council (Brit.), Special Rept. Series No. 103.

GRAM-POSITIVE NON-SPORULATING ANAEROBIC ROD-SHAPED BACTERIA OF THE INTESTINAL TRACT

III. Intra- and Extra-Group Relationships[1]

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Much attention has been directed in recent years to a group of non-sporulating anaerobes which are now known to constitute a large part of the intestinal flora of man. These organisms, both the gram-negative and gram-positive, were for a while included in the old genus *Bacteroides* of Castellani and Chalmers (1919).

The publications of Eggerth (1935), Prévot (1938) and Lewis and Rettger (1940) have fairly definitely established the validity of separating the group into two parts, based on gram reaction and cultural tests. The gram-negative species are most acceptably permitted to remain in the original genus, which is redefined to exclude the gram-positive forms. The latter are now left with no definite classification, unless serious consideration is given to the complicated scheme erected by Prévot, or the suggestions of Eggerth and of Lewis and Rettger that they be included in the genus *Lactobacillus* as an anaerobic subdivision.

It was the purpose of the present investigation to inquire into the validity of these suggestions by conducting a comparative study of the cultural, physiological and serological properties of these gram-positive forms and the apparently related oxygen-tolerating lactobacilli. As the work progressed, an investigation was made also of the respiratory mechanisms of the different organisms, with a view to further establishing their interrelationships.

The members which consitute the group that is of special interest in this report can be defined best as gram-positive, non-sporulating, non-motile anaerobic rods of fecal origin, which do not form pigment. Their nutritional requirements indicate that they are extremely fastidious, and that they are markedly stimulated by the addition of carbon dioxide to oxygen-free atmosphere in which they must be grown. They never become aerobic or microaerophilic upon continued cultivation in laboratory media, as do strains of the apparently closely related *Lactobacillus bifidus* type I, which are anaerobic on primary isolation, but become more tolerant to free oxygen on continued artificial cultivation.

The anaerobes employed in this study included a collection made up of strains received from Dr. Eggerth and Dr. Lewis, and from our own laboratory stock. In addition, numerous fresh isolations were obtained from the feces of

[1] This paper covers in part the dissertation submitted to the Graduate School of Yale University by the senior author as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

convalescent surgical patients, and in some instances from stools of normal breast-fed and bottle-fed infants. The method of isolation was the same as that described by Egerth and Gagnon (1933), except that Bacto-peptone was substituted for the Parke Davis peptone in the blood-agar medium base used by these workers.

The stock medium for carrying cultures was a modification of the glucose-cysteine agar of Lewis, Bedell and Rettger (1940). As finally used here, it had the following composition:

Bacto-tryptone	20.0 gm.
Bacto-beef extract	10.0 gm.
Bacto-agar	10.0 gm.
Bacto-dextrose	5.0 gm.
$Na_2HPO_4 \cdot 12H_2O$	4.0 gm.
Cysteine-HCl	0.5 gm.
Distilled water	500.0 ml.
Beef infusion	500.0 ml.

As a rule, 0.5 ml. of a 1:100 alcoholic solution of methylene blue was added to a liter of the medium as an oxidation-reduction indicator. The medium was dispensed in the desired quantities and sterilized by autoclaving at 120° C. for 20 minutes.

In spite of the necessity of transferring the cultures in this medium every fortnight, it was more suitable for the given purpose than the brain and egg-meat media used by previous workers. The older media enabled the organisms to remain viable for longer periods of time, but they were difficult to prepare, were easily contaminated, and did not allow ready determination of bacterial growth.

Gram-staining was carried out on 24–48 hour cysteine-glucose agar stab cultures. The procedure was one used in the laboratory of the Baltimore City Hospital and introduced into this laboratory by Dr. Mary I. Bunting.

Staining solution:

Saturated alcoholic solution of gentian violet	15 ml.
Hexyl resorcinol (Sharp and Dohme, S.T. 37)	85 ml.

Mordant solution (Burke, 1922):

Potassium iodide	20 gm.
Iodine	10 gm.
Distilled water	100 ml.

Stain for one minute, apply mordant for one minute, decolorize with acetone and counterstain with one per cent aqueous safranin.

Preparations made according to this method were more satisfactory than those obtained by other procedures. Reactions were clear-cut and the older cultures retained their gram-staining character for longer periods of time than when other staining procedures were followed.

Oxygen and carbon dioxide relationships were determined by observing the development of the organisms under different atmospheric conditions, using the cysteine-glucose medium and serum-agar plates. Anaerobic conditions were secured with the technique described by Weiss and Spaulding (1937). The

influence of carbon dioxide was determined by introduction of the gas into sealed cans containing the cultures.

For the sporulating tests 0.2 ml. of a 48-hour broth culture was added to five ml. of fresh broth, and the suspension heated at 100°C. for 10 minutes. This was followed by incubation at 37°C. for a week under anaerobic conditions.

Motility studies were made by the hanging drop method. The organisms were grown in cysteine-glucose broth, as in the sporulation tests, and examined over a period of five days.

Colonial morphology was studied at different stages of development on serum-agar and on the cysteine-glucose agar. The serum-agar was the blood agar described in the isolation technique, supplemented with three per cent inactivated human serum. As the reaction of this medium was markedly altered by the addition of the serum, it was necessary to readjust it to pH 6.8 with sterile ten per cent NaOH. The colonies were examined macroscopically and under the low power objective.

Carbohydrate fermentation reactions were determined by the methods described by Eggerth and by Lewis and Rettger. These procedures appeared to check each other quite well, with only occasional minor differences. Bacto-peptone was substituted for the Parke Davis peptone in the Eggerth medium.

The cysteine-glucose broth cultures used in the motility study were incubated further and tested with Kovac's reagent for the presence of indole. The addition of 0.05 per cent ferric ammonium citrate to the cysteine-glucose agar made this an excellent medium for hydrogen sulphide detection.

For determining the reactions in milk, skim milk containing bromcresol-purple as an indicator was employed.

Gelatin liquefaction tests were made in the media advocated by Eggerth and by Lewis and Rettger. There was a close agreement between the results obtained in the two media.

A study was made of the gram reaction and cellular and colonial morphology of over 100 recently isolated strains of the gram-positive non-sporulating anaerobes and forty stock cultures of known lactobacilli. All of the strains observed were non-motile. All revealed a wide range of pleomorphism, with changes in cultural environment and treatment generally. Rapid transfer invoked the most rapid changes in cell morphology; many bizarre forms were produced which were scarcely recognizable as bacteria. No attempt was made to describe the various forms. On the other hand, the colonial shape and structure of the various organisms remained constant. The colonies of the oral lactobacilli, which are not reproduced here, resemble the anaerobic forms in that they have entire edges; but they could not be mistaken easily for the anaerobes, because they were much larger, more viscid, and as a rule more raised than the latter. Furthermore, the colonies of the anaerobic organisms were quite granular and could be moved about whole on the plate more readily than the others. Indeed, colonial morphology is one of the more important criteria in the differentiation of the anaerobic organisms from the known lactobacilli.

Weiss and Rettger (1938) distinguished two types of *Lactobacillus bifidus*. Their type I is the classical *L. bifidus* of Tissier; it is an obligate anaerobe only on primary isolation, while their type II is described as a persistently strict anaerobe, no matter how long it is cultivated on laboratory media. The latter form was concluded to be identical with the *Bacteroides bifidus* of Castellani and Chalmers, *Bacterium bifidum* of Orla Jensen (1919) and *Bacteroides bifidus* Group 2 of Eggerth (1935). Our present observations showed that the "*Bacteroides*" species of Eggerth, the types A1, A2 and A3 of Lewis and Rettger, and our own isolations had the same oxygen relationships as *L. bifidus* type II. These forms continued as anaerobes over the entire period of observation, whereas several cultures classed as type I lost their anaerobic nature and became microaerophilic on continued cultivation in the laboratory. All strains, aerobic or anaerobic, were markedly stimulated by the addition of carbon dioxide to their gaseous environment.

Carbohydrate reactions were of value only to demonstrate homogeneity among the anaerobic and oxygen-tolerating organisms studied. All strains fermented glucose; and almost all were able to attack galactose, mannose and lactose. On the other hand, very few strains fermented arabinose, xylose or rhamnose, or any of the more complicated glucosides, such as amygdalin. Dulcitol, sorbitol and inositol and the sodium salts of the polycarboxylic acids were not attacked by any of the organisms. Failures to attack a carbon source were correlated with the results subsequently obtained with the dehydrogenase test in which the same substances were used as hydrogen donators. It was impossible to draw any distinct line here of cleavage of species or groups, as the variable carbohydrate reactions presented a picture too confused to be unravelled. Twenty-six carbohydrates and related compounds were used in the fermentation tests.

Most of the known (facultatively aerobic) lactobacilli fermented milk, with the production of abundant acid and a firm curd. Many of the anaerobic forms reacted similarly, but a number of them appeared unable to grow in the milk medium used. This was in agreement with the experiences of Lewis and Rettger, and of Eggerth. Only a few strains liquefied gelatin. Very few produced indole. Hydrogen sulphide was at no time demonstrable. No definite subgroups could be established on the basis of any of these criteria.

RESPIRATORY ENZYMES

This part of the investigation was planned to inquire into the nature of the respiratory mechanism of the gram-positive non-sporulating anaerobic rods, and to compare this with the system possessed by the lactobacilli proper and anaerobic forms belonging to other groups. The systems investigated included the flavin or flavin-like compounds, cytochrome oxidase, peroxidase, catalase and the dehydrogenases. In a further attempt to uncover the processes involved in the oxidation-reduction reactions of the cells a study was made of the effect of certain cell poisons on the amount and distribution of growth in agar shake cultures.

The first part of this study was an attempt to extract fluorescent substances from washed suspensions of the cells. The procedure followed was that of Pett (1935); this consisted in extracting washed cells with different concentrations of alcohol, and with acetone The solvents were in turn extracted with chloroform, which removed most of the fluorescent material. Illumination of these chloroform extractions in visible light caused a breakdown of any riboflavin that was present to lumiflavin. The fluorescence of these substances could then be detected by examination of the solutions under a mercury vapor arc lamp or any other suitable source of ultra-violet rays. All of the forms studied, with the exception of the negative control organism, *Proteus* sp., revealed the presence of fluorescent substances. The organisms studied included, besides the non-sporulating anaerobes, oxygen-tolerating lactobacilli, clostridia and yeasts.

The fluorescence observed was not identical with that described by Pett or that seen in a stock solution of riboflavin used as a control. The extracted material was bluish, the control greenish. Numerous workers have attempted to explain such deviations from the conventional greenish fluorescence. The present material differed in some respects from the pigments previously described. The extreme dilution of our extracts may have influenced the type of fluorescence produced and the degree of degregation brought about by the extraction-illumination processes, thus altering the structure of the fluorescing material.

The search for the presence of cytochrome oxidase, heat-stable peroxidases and catalase in the different organisms resulted in negative findings, and hence is discussed only briefly here. The method employed for cytochrome oxidase and peroxidase was that described by Keilin (1928–29), which depended upon the oxidation of paraphenylene-diamine-hydrochloride and resultant blue coloration. The catalase test was the simple gas evolution detection method employed as a qualitative test by various workers; three per cent hydrogen peroxide was flooded over young actively growing plate cultures. In the present study the medium employed was the inactivated serum-agar described earlier in this paper. Catalase could not be demonstrated in any of the gram-positive anaerobes, nor in any of the known lactobacilli tested. On the other hand, the corynebacteria studied produced a very active catalase.

Studies on the dehydrogenating enzymes of this group were carried out in a further attempt to secure data of taxonomic value. Carbohydrates were selected as substrates, as these organisms are primarily saccharolytic in nature. The method of determination was a modification of the classical anaerobic jar technique of Thunberg. This procedure was described by Casman and Rettger (1933). The washed cells, chemical substrate, dye and buffer are mixed in test tubes set in an anaerobic jar (a Hempel desiccator is very convenient) in such a manner as to be visible from the outside. While the reaction begins immediately, decolorization does not become apparent until the jar has been completely exhausted with a Hy-Vac or other powerful pump. Preheating of the jar to incubating temperature and rapid exhaustion make the method virtually a quantitative one, especially with bacterial cells which react as slowly as those

under observation do. Normal reaction time, even for the most favorable substrate, is usually two hours or more under the conditions of the present tests. After many preliminary trials the following reagents and experimental conditions were adapted:

0.25 ml. of 1:6500 aqueous solution of methylene blue (metal-free)
0.25 ml. of 0.04 molar solution of the substrate to be studied
0.50 ml. of cells suspended in buffer in a concentration equal to twice the number of cells necessary to produce a nephelometer 5.0 suspension.

Final volume	1.0 ml.
Temperature	45°C.
Atmosphere	nitrogen
Initial and final reaction	pH 6.0
Time	3 hours

The cell suspensions were prepared by harvesting the growth from 24 to 48 hour cultures on the serum-agar plates described above. These plates were inoculated by flooding with young glucose-tryptone broth cultures and immediately removing the excess inoculum. The plates were incubated under suitable conditions, and as soon as a sufficient growth was secured the cells were harvested, washed in saline and resuspended in buffer solution. Washing was carried out but once, since further treatment tended to impair the activity of the cells. All suspensions had to be used within a few hours after their preparation, since their activity fell off rapidly even at refrigerator temperature. Controls were set up of substrate-free dye-cell mixtures.

The buffer solution employed was a Clark and Lubs phosphate buffer made up from 50 ml. of a 0.2 M $\cdot$ KH_2PO_4 solution to which was added sufficient 0.2 N sodium hydroxide to produce the desired reaction, in this case pH 6.0; the entire solution was then brought up to 200 ml. with distilled water. The pH values secured were checked colorimetrically and electrometrically; they were affected little by the addition of the other reagents used in the tests. The buffer solution was very close to isotonic for human erythrocytes; it showed no evidence of causing lysis of the bacterial cells.

Twenty-six chemical substrates were employed. Inasmuch as the reactions with the anaerobic and oxygen-tolerating groups of bacteria were quite similar, they are summarized briefly in tables 1 and 2.

The results show a more or less definite relationship between the dehydrogenating activity of the various groups of gram-positive non-spore-producing rods studied, and indicate that, on the whole, similar reactions may be obtained for the various groups with the same substrates.

Since many of the chemical substances which modify cellular metabolism do so by interfering with the enzyme systems affecting cellular oxidations, a study was made of the effect of cell poisons on the growth of the cultures, with the hope of obtaining further information regarding the respiratory mechanism of these groups. Because potassium cyanide yielded the most fruitful results, work with this agent alone is presented here. Following the lead of Burnet

(1927) and Braun and Guggenheim (1932), experiments were carried out in which the amount and distribution of growth was observed in a basal medium to which varying concentrations of this cell poison had been added.

TABLE 1

Dehydrogenase reactions of non-sporulating gram-positive rods

SUBSTRATES USED	ANAEROBES A1 AND A2	BIFIDUS TYPES I AND II	ACIDOPHILUS CULTURES	ORAL LACTOBACILLI
Arabinose	–/15	–/4	–/4	–/4
Cysteine*	15/–	4/–	4/–	4/–
Cellobiose	11/4	4/–	3/1	2/2
Dulcitol	2/13	–/4	2/2	1/3
Fructose	14/1	3/1	4/–	3/1
Galactose	15/–	4/–	4/–	4/–
Glucose	4/–	4/–	4/–	4/–
Glycerol	8/7	3/1	3/1	3/1
Inositol	7/8	–/4	1/3	1/3
Inulin	1/15	–/4	–/4	–/4
Lactose	6/9	1/3	2/2	4/–
Maltose	10/5	3/1	2/2	2/2
Mannitol	14/1	3/1	4/–	4/–
Mannose 5	8/7	1/3	4/–	4/–
Melezitose	5/10	1/3	3/1	2/2
α-Methyl mannoside	1/14	–/4	–/4	–/4
Raffinose	5/10	–/4	–/4	–/4
Rhamnose	1/14	–/4	–/4	–/4
Salicin	6/9	2/2	2/2	3/1
Sorbitol	8/7	2/2	3/1	4/–
Sucrose	7/8	3/1	4/–	2/2
Xylose	–/15	–/4	–/4	–/4
Fumarate	–/15	–/4	–/4	–/4
Succinate	–/15	–/4	–/4	–/4
Control†	–/15	–/4	–/4	–/4

First figure indicates the number positive. Second figure the number negative. For example 3/6 means that 3 strains were positive, and 6 negative in the given dehydrogenase test.

* Control to check efficiency of the anaerobic method.

† Cell suspension plus dye, and twice the cell suspension plus dye, without substrate.

The basal medium used in these experiments was the semi-solid glucose-tryptone medium of Lewis, Bedell and Rettger described earlier in this report.[2] Agar shake tubes containing the potassium cyanide in the desired concentra-

[2] A serum-peptone semi-solid agar was also used which gave results quite similar to those obtained in the glucose-tryptone medium. Because this medium became opaque upon development of the cultures, the results are omitted here.

TABLE 2

Reaction of different substrates in the dehydrogenating action of non-sporulating gram-positive rods

Substrates serving as H acceptors:	
d-Fructose	
d-Galactose	
d-Glucose	
d-Mannitol	
Substrates failing to act as H acceptors:	
d-Arabinose	Sodium citrate*
Inulin	Sodium fumarate
α-Methyl mannoside	Sodium oxalate*
l-Rhamnose	Sodium succinate
d-Xylose	Sodium tartrate*
Substrates giving varied reactions with the different members of the group of bacteria under study:	
Cellobiose	d-Mannose
Dulcitol	Raffinose
Glycerol	Salicin
l-Inositol	d-Sorbitol
Lactose	Sucrose
Maltose	Melezitose

* The results obtained with these substrates are not included in the data reported in the preceding chart, as they were carried out under conditions somewhat different from those described.

TABLE 3

The influence of cyanide on the distribution of growth in cultures of various bacterial forms

ORGANISM	CONCENTRATION OF POTASSIUM CYANIDE USED					
	1:500	1:1000	1:2000	1:5000	1:10,000	None
Anaerobe No. 1 of our isolation	C	CD	CDE	CDE	CDE	CDE
Anaerobe No. 2 of our isolation	C	CD	CDE	CDE	CDE	CDE
Anaerobe No. 1 Eggerth isolation	–	C	CDE	CDE	CDE	CDE
Anaerobe No. 2 Eggerth isolation	?	C	CD	CDE	CDE	CDE
L. bifidus type II	–	C	CD	CDE	–	CDE
L. bifidus type I	–	C	CD	CDE	CDE	CDE
L. acidophilus	BCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
L. bulgaricus	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
L. pentoaceticus	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
Escherichia coli	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
Salmonella pullorum	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
Clostridium sporogenes	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
Bacillus megatherium	A	A	A	A	A	A
Bacillus mesentericus	A	A	A	A	A	A
Staphylococcus aureus	BCDE	BCDE	BCDE	BCDE	ABCDE	ABCDE

? = questionable growth. – = no growth. The letters indicate growth in the respective zones to which they refer.

tions were employed. The stratification described below occurred in tubes that were heavily seeded from tryptone broth cultures of the respective organism. The inoculum finally adopted was 0.1 ml. of a 24 to 48 hour culture. The final pH of the inoculated tubes was brought to pH 7.0–7.2.

Not only was inhibition of growth observed in the cyanide sensitive tubes, but also an uneven distribution of growth through the tubes, as is shown in table 3, with the aid of the accompanying schematic drawing (fig. 1).

There was definite growth inhibition of the positive non-sporulating anaerobes and *L. bifidus* type I in the deepest layers (D and E) of the tubes containing

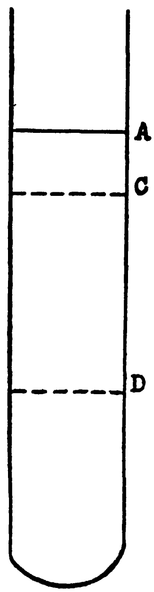

FIG. 1. ILLUSTRATING DIFFERENT ZONES

A = surface growth. *B* = area just below surface in which the dye is not reduced (5–10 mm. in depth). *C* = border between the reduced and oxidized medium. *D* = approximately 30 mm. depth below the line marked *C*. *E* = remainder of the tube.

the KCN in the higher concentrations, namely 1:500, 1:1000 and 1:2000. This was in sharp contrast to the results obtained with *Lactobacillus acidophilus*, *L. bulgaricus*, *L. pentoaceticus*, *Escherichia coli*, *Salmonella pullorum* and *Clostridium sporogenes*, all of which grew throughout the tubes, except *L. acidophilus*, which failed to produce a surface growth. The organisms differed also from *Bacillus megatherium* and *Bacillus mesentericus*, which developed a surface growth only, and from *Staphylococcus aureus*, which produced uniform growth throughout the tubes in all cyanide dilutions except surface growth in dilutions 1:500 to 1:5000.

Thus the results indicate a definite cyanide-sensitive system in the anaerobic organisms and *L. bifidus* type I. We may conclude that this system is not flavin, as flavins are not known to be injured by cyanide. While these forms are defi-

nitely inhibited by the higher concentrations of KCN, they are apparently indifferent to the weaker solutions, and may be regarded therefore as having both a KCN-sensitive and insensitive system.

In contrast to the dual reaction of the anaerobes and *L. bifidus* type I to KCN, *L. acidophilus*, *L. pentoaceticus*, *E. coli*, *S. pullorum* and *C. sporogenes* were insensitive to all of the KCN dilutions employed, except *L. acidophilus* in the highest concentration.

The results obtained here support in a large measure those of Burnet (1927) and of Braun and Guggenheim (1932).

The use of indole and phenol as possible cell poisons revealed that the gram-positive intestinal anaerobes were as resistant to these agents as the known oxygen-tolerating intestinal lactobacilli.

SEROLOGICAL APPROACH

Agglutination, agglutinin absorption, precipitation and complement fixation tests were conducted on the non-sporulating gram-positive organisms under special study here and several well-known lactobacilli. Only the complement fixation method furnished information that could be of any value. Agglutination reactions showed such a high degree of strain specificity as to make the results worthless. The agglutinin absorption and precipitation methods were disappointing also; the former, because of our failure to develop agglutinating sera of very high titre, and the precipitin test because not sufficient antigen could be extracted from the cells to serve the purpose. On account of the more or less indifferent results obtained with these three methods, considerable attention was given to the fixation method.

Previous studies of complement fixing relationships of gram-positive rods have centered principally around the diphtheria and diphtheroid groups. Bluhdorn (1910) employed extracts of *L. acidophilus* and *L. bifidus* as antigens in complement fixation reactions. He was able to observe inter-relationships between different strains of these organisms, but was able also to demonstrate species differences. Jötten (1922) observed group specificity in some of the strains, and not in others. Kulp and Rettger (1924) conducted complement fixation studies on autolyzed cells of *L. acidophilus*, *L. bifidus* and *L. bulgaricus*. Reactions obtained with heterologous and homologous immune sera were sufficiently strong to relate these organisms all to one and the same serological group. Hunt and Rettger (1930) showed marked relationships among grain and soil lactobacilli by this procedure.

The bacterial antigens employed in the present study were freshly washed suspensions of cells made up to a turbidity of 2 on the McFarland nephelometer scale. The organisms were grown on serum agar plates (pH 6.6 to 6.8)[3] incubated at 37°C. for 24 to 48 hours. The final saline suspensions were heated to

[3] When the medium was adjusted to a reaction above pH 6.8, the resultant antigen was found to possess a highly anti-complementary titer. Older cultures were useless for the same reason. In one instance an antigen prepared from an old (five days) culture showed anticomplementary activity in dilutions of 1:10,000,000.

60°C. for one hour. Since antigens which were kept longer than 24 hours, even in the refrigerator, developed anti-complementary activity, it was necessary to prepare fresh suspensions daily. The antigens so prepared were titrated

TABLE 4

Complement fixation tests

ORGANISMS	SERUM DILUTION*				CONTROLS	
	(1) Undiluted	(2) 1:2	(3) 1:4	(4) 1:8	(5) Antigen	(6) Serum
Intra-group relationships						
Anaerobic strains isolated from same stool						
(1)	++	++	++	–	–	–
(2)	++	++	++	–	–	–
(3)	++	++	++	+	–	–
Anaerobes from other sources						
Lewis types A_1 and A_2						
(1)	++	–	–	–	–	–
(2)	++	++	–	–	–	–
(3)	++	++	++	–	–	–
(4)	++	++	–	–	–	–
(5)	++	++	++	++	–	–
L. bifidus type II						
(1)	++	++	++	–	–	–
(2)	++	++	++		–	–
(3)	++	++	++	–	–	–
Inter-group relationships						
L. bifidus type I						
(1)	++	–	–	–	–	–
(2)	++	–	–	–	–	–
L. acidophilus						
Wickerham strain	++	++	–	–	–	–
Sherman strain	++	++	–	–	–	–
Oral lactobacilli						
(1)	++	++	–	–	–	–
(2)	++	++	–	–	–	–
(3)	++	–	–	–	–	–
Döderlein's bacillus	++	++	++	–	–	–
L. bulgaricus	–	–	–	–	–	–

++ = complete fixation of complement. + = partial fixation of complement. – = no fixation of complement.

* This particular serum was prepared against strain K-13. Similar results were obtained with other strains.

for hemolytic and anti-complementary activity. The specific complement-binding power of several representative preparations of these antigens for homologous antisera lay in the neighborhood of 1:12,000 (as compared to anti-

complementary (non-specific) titers in the same preparation of only 1:50 and 1:125). For this reason it was not deemed necessary to check the titer in all preparations. For the test dose of antigen one-third of the lowest dilution of reagent revealing anticomplementary activity was adopted. This was usually around 1:150 to 1:400.

The antisera employed were prepared in the usual manner. They were inactivated at 55°C. for thirty minutes, and preserved in the lyophilized state, in order to prevent objectionable increase in anticomplementary activity.

The hemolysin against sheep's red cells was developed in rabbits by the usual procedure. The complement was secured by cardiac puncture of two or more male guinea pigs. The red cells were prepared in 5 per cent suspension and mixed with the complement immediately before use, to make a final concentration of 2.5 per cent centrifuged cells and two units of amboceptor.

An attempt was made to determine the relationship of the various anaerobic strains, first to each other, second to the facultative intestinal lactobacilli, third to the oral and other lactobacilli, and finally to organisms outside of the lactobacillus genus. The data on the gram-positive non-spore formers are summarized in table 4.

Tests made on members of the subtilis group, several coccus forms, *Escherichia coli*, *Salmonella pullorum*, *Proteus vulgaris* and several *Bacteroides* (gram-negative) organisms gave negative fixation reactions. *Corynebacterium diphtheriae*, on the other hand, was definitely positive to the test.

The data presented in table 4 indicate the existence of a more or less definite relationship between the different species or groups of gram-positive, non-sporulating bacteria of various origin; also that the strains of the anaerobic division are more closely related to each other than they are to the other lactobacilli studied.

SKIN SENSITIVITY TESTS

Further study led to an inquiry into the specificity of local skin reactions which were observed earlier in sensitized rabbits that had been injected locally with antigenic cellular preparations from homologous cultures.

Bacterial cells were employed in both the sensitization and reacting doses, since previous experiments had shown that extracts of the cells were non-antigenic. Over 350 tests were conducted on 30 different rabbits. The sensitizing doses were as a rule a series of two or three graded (0.5 to 1.0 ml.) suspensions of the test organisms having a density of 2.0 in the McFarland nephelometer scale. The injections were made in the marginal ear vein; they were timed with reference to the skin tests, which followed after a week to ten days after the last injection. The antigens were as a rule suspensions of live cells harvested from 24–28 hour growths on serum agar plates. The few experiments conducted with heat-killed antigens showed that the heating did not render the cells visibly less antigenic. All shocking injections were made intradermally with 0.25 ml. of live cell suspensions having a density of 2.00. Control tests were conducted with sterile broth, milk, human serum and saline solution.

The lesions produced as a result of the intradermal injection of the antigen following the sensitizing doses appeared within three days. Pseudo-reactions which disappeared soon were frequently observed at the end of twenty-four hours. These reactions were non-specific and were believed to be due to the irritant action of the mass of cells present in the shocking dose. A typical positive reaction may be described as follows. The bleb produced by the intradermal injection of the antigen disappeared in a short time, and no change could be noted at the site of injection for 18–20 hours. Soon after this the skin

TABLE 5

Skin reactions to cellular bacterial antigens in rabbits sensitized to gram-positive non-sporulating anaerobic rods

CULTURES	NUMBER OF		REACTION IN RABBITS	
	Strains	Tests	Normal	Sensitized
Anaerobic gram-positive rods				
Groups A1, A2 and A3 of Lewis	8	63	0	++
Anaerobic gram-positive rods from Eggerth	3	25	0	++
Microaerophilic "*Bacteroides*" *bifidus* from Eggerth	2	7	0	++
Anaerobic rods of our own isolation	12	49	0	++
Aerobic oral lactobacilli	4	79	++*	++
Aerobic intestinal lactobacilli	4	16	−	−
Aerobic grain and dairy lactobacilli	2	16	±	+
Corynebacteria	4	16	(delayed lesions in toxin-producing strains)	
Anaerobic gram-negative rods (Bacteroides) of Eggerth	3	7	−	−
Other gram-positive and gram-negative organisms†	14	52	−	−
Saline, broth, serum, etc.	5	19	−	−

* It will be noted that the oral strains of lactobacilli produced lesions in the skin of the rabbits, whether the animals had been sensitized or not. Because of this action the value of the test is limited when applied to this part of the lactobacillus group. The same was observed also, but to a lesser extent, with the dairy and grain forms. Killed antigens reacted similarly, but to a lesser degree.

† Including *E. coli*, *S. pullorum*, *Bacillus subtilis*, *Micrococcus ureae* and *Staphylococcus aureus*.

became reddened and infiltrated, so that it could be taken between the fingers in a fold; it also became definitely thickened. The swelling and redness continued to increase, and reached their heighth in about forty-eight hours, when the site of injection presented a swollen area of about fifteen millimeters in diameter surrounded by a second reddened zone of approximately equal diameter. The lesion seemed tender, although the animals evidenced no discomfort when moving about or lying down. In a few severe cases these lesions became necrotic and broke down in the center, healing taking place only after a long period of time, usually at least 2 or 3 weeks.

This reaction was typical for all homologous strains employed. There was a definite grading in severity among reactions produced by heterologous strains in both homologous and heterologous groups. However, the intergroup separations were much more marked than the intragroup alignments. The group relationship is indicated in table 5.

DISCUSSION AND SUMMARY

The present investigation has served to further establish two facts which are of paramount importance; first, that the gram-positive non-sporulating anaerobic bacteria of intestinal origin, which were the chief object of study here, hold no legitimate place among the gram-negative non-spore-producing anaerobes of similar host origin, the so-called "bacteroides" group, and second that they constitute a homogeneous group of their own.

The gram-staining properties, cell morphology, and cultural and physiological characteristics link these anaerobic organisms definitely with the lactobacilli in general, particularly the intestinal forms, *Lactobacillus acidophilus* and *L. bifidus*. The presence of certain respiratory enzymatic agents (flavin or flavin-like substances and dehydrogenases) and the absence of others, namely catalase, cytochrome, oxidase and peroxidase also reveal a close relationship.

Potassium cyanide exerted a poisoning influence on all of the species or subgroups studied, but the action was much more pronounced in the cultures of the anaerobes than in those of the facultative aerobes.

Resistance to the action of indole and phenol was decidedly greater among the anaerobes in question and in the related intestinal organisms than in the lactobacilli of non-intestinal origin.

Colony size and morphology served, on the whole, to separate the different subgroups from each other, but they can not always be employed as important classification criteria, even though the colonial characteristics of the different species were reasonably constant.

The serological approach to the differentiation problem offered little of real value. Complement fixation tests pointed to definite heterogencity among the different groups, but this was limited and served only in a broad way to separate the gram-positive intestinal anaerobic forms from the facultative aerobes in the group at large, and from true intestinal bacteroides.

The skin test (Arthus phenomenon) was in fair agreement with the complement fixation test; it has some merit in separating the intestinal gram-positive anaerobes from the facultative groups of various origin. Proper controls and exact titration of reagents are highly essential in the conduct of this test.

Pathogenicity tests on mice and rabbits gave negative results, except in rabbits that were injected with oral lactobacilli; these cultures caused marked local skin lesions.

Relation of free oxygen to the growth of members of the *Lactobacillus* genus under present study constitutes the only criterion of far-reaching importance in the separation of the organisms in the group as a whole into component parts

or subgroups, particularly when it is supplemented by knowledge of the origin of the organisms under observation.

Inquiry into the relationship of the strictly anaerobic forms with Eggerth's *Bacteroides bifidus* (Group 2) and Orla-Jensen's *Bacterium bifidum* has forced upon us the conclusion that these two organisms and the gram-positive anaerobic rod forms receiving special attention here are so intimately related to each other that they should be regarded as one and the same species, and that the name suggested by Weiss and Rettger (1938) for intestinal anaerobic gram-positive non-sporulating rods, namely *Lactobacillus bifidus* type II, be applied to the group.

Note: During the course of catalase study two strains of the intestinal gram-positive non-sporulating anaerobes gave positive tests for this enzyme. These strains were set apart for future investigation. Quite recently they were found by Dr. H. Albert Barker, visiting fellow in our laboratory, to produce large quantities of volatile acids, chiefly propionic, in appropriate carbohydrate media. This observation and the demonstration by us of catalase activity suggest a close relationship of these organisms with the propionic acid bacteria, rather than the lactobacilli. This question awaits further study.

REFERENCES

Blühdorn, Kurt 1910 Untersuchungen über den *Bacillus bifidus communis* und den sogen. *Bacillus acidophilus* (*Streptobacillus faecalis*). Jahrb. Kinderheilk., **72**, 693–704.

Braun, H., and Guggenheim, K. 1932 Ueber Atmungstypen bei fakultative aeroben pathogenen Bakterien. Zentr. Bakt. Parasitenk. I. Orig., **127**, 97–104.

Burke, Victor 1922 Notes on the gram stain with description of a new method. J. Bact., **7**, 159–183.

Burnet, F. M. 1927 The action of cyanides on bacteria. J. Path. Bact., **30**, 21–38.

Casman, E. P., and Rettger, L. F. 1933 Limitations of bacterial growth at higher temperatures. J. Bact., **26**, 77–123.

Castellani, Aldo, and Chalmers, A. J. 1919 Manual of tropical medicine, 3rd edition, p. 2436 et seq., London, Ballière, Tindall and Cox.

Eggerth, Arnold, and Gangon, H. H. 1933 The bacteroides of the human feces. J. Bact., **25**, 389–413.

Eggerth, Arnold 1935 The gram-positive non-spore-bearing anaerobic bacilli of human feces. J. Bact., **30**, 277–299.

Hunt, G. A., and Rettger, L. F. 1930 A comparative study of members of the *Lactobacillus* genus, with special emphasis on lactobacilli of soil and grain. J. Bact., **20**, 61–83.

Jötten, J. W. 1922 Vergleiche zwischen dem Vaginalbazillus Döderleins und dem *Bac. acidophilus* des Säuglingsdarmes. Arch. Hyg., **91**, 142–157.

Keilin, D. 1928–29 Cytochrome and respiratory enzymes. Proc. Roy. Soc., (London) B., **104**, 206–251.

Kulp, W. L., and Rettger, L. F. 1924 Comparative study of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. J. Bact., **9**, 357–394.

Lewis, K. H., Bedell, Margaret, and Rettger, L. F. 1940 Non-sporulating bacteria of the intestinal tract. II. Growth-facilitating factors. J. Bact., **40**, 309–320.

Lewis, K. H., and Rettger, L. F. 1940 Non-sporulating anaerobic bacteria of the intestinal tract. I. Occurrence and taxonomic relationships. J. Bact., **40**, 287–307.

Orla-Jensen, S. 1919 The lactic acid bacteria. Mém. acad. roy. sci. Danemark, **5**, 81–196 (with 51 unnumbered plates.)

PETT, LIONEL B. 1935 Lactoflavin in micro-organisms. Biochem. J., **29**, 937–944.

PRÉVOT, A. P. 1938 Études de systematique bactérienne. III. Invalidité du genre Bacteroides Castellani et Chalmers démembrément et reclassification (1) Ann. inst. Pasteur, **60**, 286–307.

TISSIER, HENRY 1900 Étude sur la flore intestinale normale et pathologique du nourrissons. Thèse de Paris. (cited by Weiss and Rettger (1938) and others.)

WEISS, J. E., AND RETTGER, L. F. 1938 Taxonomic relationships of *Lactobacillus bifidus* (*B. bifidus* Tissier) and *Bacteroides bifidus*. J. Infectious Diseases, **62**, 115–120.

WEISS, J. E., AND SPAULDING, E. H. 1937 A simple method of obtaining effective anaerobiosis. J. Lab. Clin. Med., **22**, 726–728.

A STUDY OF THIOBACILLUS THIOOXIDANS WITH THE ELECTRON MICROSCOPE

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Thiobacillus thiooxidans, an obligate autotrophic sulfur-oxidizing bacterium, has received considerable attention because of the interest and importance attached to a knowledge of its physiology. In the original description of the organism (Waksman and Joffe, 1922) was included a photomicrograph the essential features of which are reproduced from our own cultures in figure 1. We have recently had the opportunity to examine pure cultures of this microorganism in the RCA electron microscope, using distilled water suspensions prepared and micrographed by methods previously described (Umbreit *et al.* 1941, Marton 1941). These electron micrographs (figures 2 through 6) seem to relate so directly to the original photomicrograph that we have felt that they would be of interest to bacteriologists even though the interpretations we have placed upon the evidence at hand may be modified by future study. We have repeatedly noted the forms found in these micrographs in countless preparations studied in the light microscope by a variety of staining methods. Their observation in the electron microscope illustrates, we believe, the profound effect which the latter instrument will exert upon bacteriology.

OBSERVATIONS

The cells observed seem to fall into three general types with transition stages sometimes evident. These are:

Type I (1 by 0.5μ); stainable with alcoholic crystal violet (Waksman and Joffe, 1922) or better with crystal violet followed by iodine (Umbreit *et al.*, 1941) Under staining procedures they frequently exhibit a "dipolar" appearance (fig 1, *a*) It has recently been shown (Umbreit *et al.*, 1941) that these dipolar bodies consist of a highly unsaturated fat and are involved in the process of sulfur oxidation The electron micrographs (*a* in figs. 2, 3, 4, 5) show the same type of cell as relatively opaque to the electron beam The dipolar appearance noted in the stained cells is not evident in the electron micrographs. This is due to either of two possibilities, one, that the electron beam does not distinguish between the fat and other cell constituents, or, two, that the high vacuum in the microscope has evaporated the fat from the cell Since the fat is quite volatile this would be a definite possibility and the slightly opaque granules noted in some cells of figures 2 and 3 might be regarded as residues from the evaporation.

Type II (2–3 by 0.5μ); evident in the photomicrograph in which it appears as a relatively homogenous cell of elongated form (fig. 1, *b*). Such cells might arise by a delay in cell division as seems to have been the case in certain forms noted (see *b* in figs. 2 and 3). But in others (figs. 5 and 6) a more complex structure is noted. In figure 6, particularly, the internal spiral structure, whose nature and function are unknown, is strikingly evident.

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All of these longer types are relatively opaque to the electron beam and stain almost as readily as the Type I cells.

TYPE III (1-1.5 by 0.5–0.8 μ) do not stain readily (fig. 1, *c*) and are much less opaque to the electron beam. We would regard types labeled *c* in figures 2 and 3 as representatives of this type and *ac* in figure 4 as a transition form between Type I and Type III.

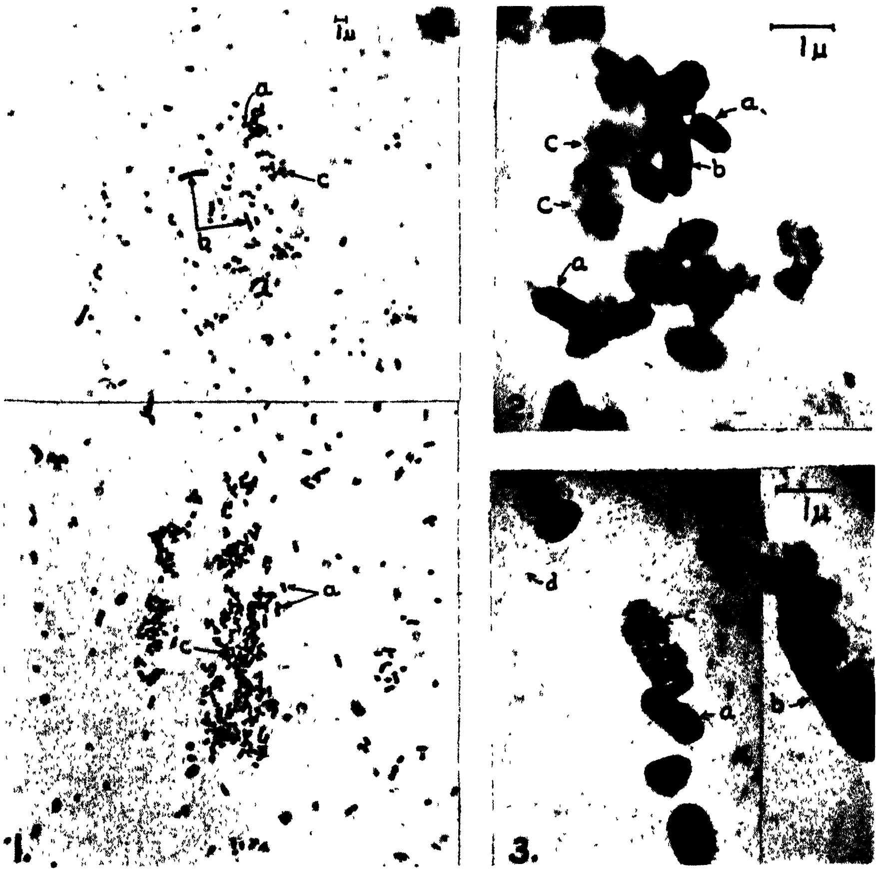

FIG. 1. THIOBACILLUS THIOOXIDANS, IN LIGHT MICROSCOPE, STAINED WITH CRYSTAL VIOLET

Top—three day culture. Bottom—twenty day culture. Original 900 ×. Enlargement 2 ×. Total magnification 1800 ×.

FIG. 2 THIOBACILLUS THIOOXIDANS, UNSTAINED, IN ELECTRON MICROSCOPE. 12,000 ×

FIG. 3. AS FIGURE 2

In addition, flagella may be noted (*d* in figs. 3 and 6) but it is probably of some significance that most of the cells are nonflagellated. In figure 4 there is a faint halo around certain cells; this we believe to be the bacterial cell-wall away from which the opaque, black, protoplasm has shrunk. (Mudd *et al.*, 1941). We regard the exceedingly faint "halo" extending for about a micron around the cells in figure 6 as an artifact developed in the preparation of the specimen.

Fig. 4. As Figure 2
Fig. 5. As Figure 2
Fig. 6. Thiobacillus Thiooxidans, Unstained, in Electron Microscope. 19,000 X

INTERPRETATION

It is obvious that any interpretation of the nature of the structures observed in these cells is decidedly premature and we therefore advance none. However, it may be of value to point out that a culture of *T. thiooxidans* is not a homogeneous entity but contains cells of all stages of growth, of all degrees of nutrition, and under environmental conditions (particularly low pH) which are decidedly detrimental to other forms. In the course of its growth on sulfur the

organism synthesizes a reserve storage product which enables it to live in the absence of oxidizable sulfur (Vogler, 1941). This supply of reserve food is sufficient for a considerable period of survival but will not last indefinitely. When it has been used up the cell dies and undergoes a slight autolysis. One might regard cells of Type I as those having a high reserve of storage products and those of III as being dead cells whose storage products had been utilized. Further work is necessary to determine whether this explanation is suitable. The structures observed in figure 6 present an interesting problem in bacterial cytology and their function and nature are matters for further study.

SUMMARY

Electron micrographs of *Thiobacillus thiooxidans* reveal that the cells possess a thin cell-wall differentiated from the internal protoplasm. The cells may be roughly divided into three categories:

1. Oval cells containing so much internal matter that they appear opaque to the electron beam.
2. Elongated cells containing less internal matter and exhibiting a wide variety of structures, granules, vacuoles, and even spirals.
3. Oval cells containing very little matter.

Flagella, which are about 17 mμ wide, occur only rarely. Comparisons of these micrographs with those of the light microscope are made and the possible physiological interpretations of the observed structures are briefly discussed.

REFERENCES

Marton, L. 1941 The electron microscope a new tool for bacteriological research. J. Bact., **41**, 397–413

Mudd, S., Polevitzky, K., Anderson, T. F., and Chambers, L. A. 1941 Bacterial morphology as shown by the electron microscope. II. The bacterial cell-wall in the genus *Bacillus*. J. Bact., **42**, 251–264.

Umbreit, W. W., Vogel, H. R., and Vogler, K. G. 1942 The significance of fat in sulfur oxidation by *Thiobacillus thiooxidans*. J. Bact., **43**, 141–148.

Vogler, K. G. 1941 Unpublished data.

Waksman, S. A., and Joffe, J. S. 1922 Microorganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur oxidizing organism isolated from the soil. J. Bact., **7**, 239–256.

OXYGEN DEMAND AND OXYGEN SUPPLY

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V. THE MULTIPLICATION CURVE

Bacteriologists have often wondered why the so-called "logarithmic growth phase," i.e., the phase of truly exponential multiplication, is so short in our culture media. With the rapidly growing aerobes and facultative anaerobes which are the common organisms for general growth studies, this phase where the cells multiply at a constant rate does not last more than 2 to 4 hours at or near the optimal temperature.

As a rule, the logarithmic multiplication curve begins to deviate from the straight line when the cell population has reached approximately ten million bacteria per ml.

The facts mentioned in chapters II and III of this treatise (J. Bact., **41**, pp. 226 & 234, 1941) give a simple explanation of this short period of constant multiplication rate. It had been shown there that with most oxygen-consuming bacteria, the supply of oxygen dissolved in the medium is exhausted by the time these bacteria have reached 2 to 10 million cells per ml. Further multiplication will proceed at the same rate only on the surface, where oxygen from the air is continually renewed, but not at the bottom where anaerobic decomposition may still provide energy for growth, but at a much slower rate. The deviation of the logarithmic multiplication curve from the straight line indicates therefore, that the dissolved oxygen in the medium is exhausted.

This can be proved very simply by comparing the same organism in an ordinary flask culture and in an aerated culture. Figure 1 shows this for *Pseudomonas fluorescens* in nutrient broth. While the ordinary culture in an Erlenmeyer-flask had a constant rate of multiplication only from 2 to 6 hours, the aerated culture, although delayed at first, multiplied at a constant rate from 4 to 12 hours. The initial delay by aeration which is a regular occurrence will be explained in chapter VI. Other examples showing that aeration greatly extends the period of a constant multiplication rate will be found in figures 3 to 6.

In order to test these assumptions in a different way, and to make the argument more convincing, a calculation has been made of the depth to which the oxygen of the air will penetrate into a growing culture with more than 10 million bacteria per ml. *Bacillus mesentericus* was grown in air-saturated peptone solution in four cultures; one was continuously aerated; one was a 200 ml. culture in a 2 liter Erlenmeyer-flask; the other two were kept under oil, one with methylene blue to indicate the moment of complete oxygen exhaustion, the other without the dye to see whether methylene blue would influence the growth or death rate. The results are shown in table 1. All data are averages of triplicate plates.

Multiplication was uniform in the last three cultures until the dissolved oxygen was used up. Then the bacteria under oil died rather rapidly at first as had been shown in chapter IV (J. Bact., **41,** p. 241) while the Erlenmeyer-flask culture continued to multiply, though the rate decreased progressively. Aeration caused the usual initial retardation, but multiplication continued, though somewhat more slowly after the 8th hour, quite rapidly until the 12th hour.

At 8 hours, the two aerobic cultures had practically the same number of cells, 16.3 and 16.8 millions per ml. The aerated culture multiplied in 2 hours to 74.7 millions, requiring 48 minutes for doubling. The bacteria on the surface of the Erlenmeyer culture must have doubled at the same rate, but at the bottom,

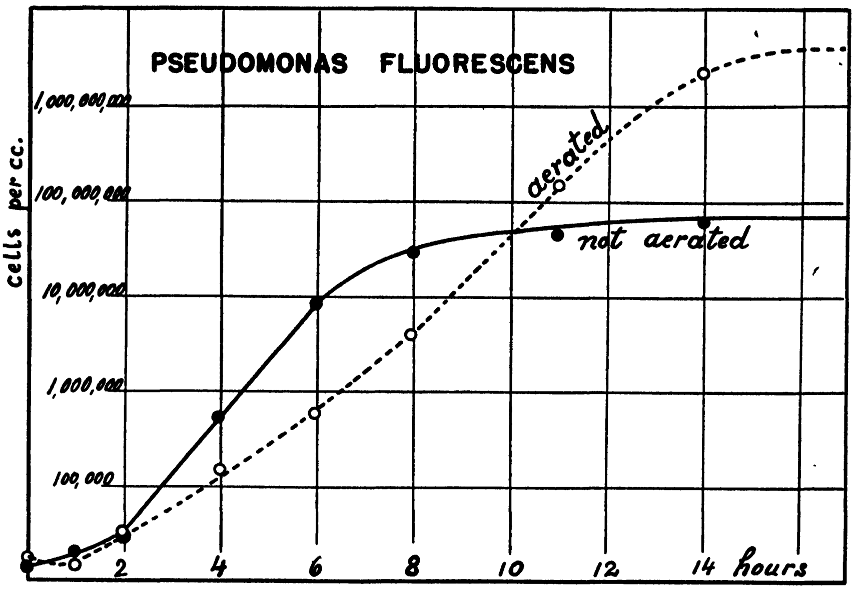

FIG. 1. MULTIPLICATION OF PSEUDOMONAS FLUORESCENS IN AN ORDINARY FLASK CULTURE AND IN THE SAME MEDIUM STRONGLY AERATED

without oxygen, they died at the same rate as the cultures under oil at the time the oxygen gave out, i.e., at least half of them died in 2 hours. Let us call the volume of culture receiving oxygen x, then the volume without oxygen is $200 - x$ as the total culture is 200 ml. In the x ml., the bacteria multiply to 74.7 million per ml. while in the rest of the culture, numbers decrease from 16.8 to 8.4 million per ml. The total number of bacteria in the culture is,

$$74.7\,x + 8.4\,(200 - x) = 18.9 \cdot 200$$
$$x = 31.7 \text{ ml.}$$

Only 32 ml. of this culture received sufficient oxygen to permit bacterial multiplication. The surface of the culture was 14 cm. in diameter, or 154 cm.[2] in surface, which shows that the oxygen penetrated only 2.05 mm. below the surface.

Assuming the same rate of multiplication for the next two hours when oxygen was provided, only 27 ml. of the culture received oxygen.

These calculations have assumed that all the oxygen came through diffusion from the surface. However, the culture had been shaken to obtain the samples for the plate counts, and there must have been convection currents carrying oxygen into the medium. In a perfectly quiet culture, the surface layer that receives oxygen from the air above must be even less than 2 millimeters thick.

While this exhaustion of oxygen explains the short period of constant multiplication rates with aerobic and facultative organisms, it does not apply to streptococci and lactobacilli which do not consume oxygen in great amounts and

TABLE 1

Multiplication of Bacillus mesentericus in 1 per cent peptone solution with different oxygen supply

TIME	BACTERIA PER CUBIC MILLIMETER			
	Continuously aerated	In Erlenmeyer flask, quiet	Covered with oil, methylene blue	Covered with oil, no methylene blue
hours				
0	61.7	61.7	61.7	61.7
1	58.3		101.7	68.7
2	91.7		107.3	91.0
3	140.0	190.0	340.0	263 0
5	650.0	1,943	4,870	4,470
5.5			8,000	7,500
6	1,770	6,500		
6.5			7,970	2,197
7	5,500	10,870		
7.5			4,160	1,907
8	16,300	16,800		
10	74,000	18,930	3,087	890
11			2,210	1,770
12	120,500	20,500	2,385	1,700
13		25,000	1,800	1,450
26	245,000	44,500	1,600	1,100

which are not speeded up by aeration (see e.g. fig. 5). Their early retardation of multiplication is not due to oxygen, and will be treated in a separate paper.

VI. FINAL CROPS AND THE AUTOTOXIN THEORY

That the final crop of microorganisms in a given culture medium may be greatly increased by aeration, has been found empirically by the Vienna bread yeast manufacturers who discovered this fact some 40 years ago. Two other processes use aeration on an enormous scale, namely vinegar manufacture and sewage disposal by trickling filters or contact beds. In both processes, the proof of a larger microbial crop through aeration is only indirect, but none the less convincing.

The application of this long-known fact to laboratory technique has been quite

slow. Among the first to show the great increase by aeration in the crop of facultative anaerobes were Winslow, Walker, and Sutermeister (1932). They grew *Escherichia coli* in broth with and without lactose, and found that aeration caused at first a slight delay, but soon afterwards a more rapid growth, a longer period of constant multiplication and a final cell population 5 to 10 times as large as that in unaerated controls. Since then, aeration has been used frequently when large quantities of cells were wanted.

The following experiment does not contribute any new facts in regard to cell populations, but is mentioned to explain a point in technique. A *Mycoderma* which oxidizes lactic acid rapidly was cultivated in a mineral medium with NH_3 as only source of nitrogen, and lactic acid as only source of carbon. When the original amount of 1 per cent lactic acid was depleted, more concentrated acid was added.

Table 2 shows that multiplication without aeration had soon reached its peak while aeration increased the cell concentration enormously. An interesting and

TABLE 2

Effect of aeration upon a mycoderma oxidizing lactic acid

	NON-AERATED CULTURE		AERATED CULTURE		
			Total acid consumed	Cells per cm^3	
	Total acid consumed	Cells per cm^3		Liquid	Foam
	gm./liter		*gm./liter*		
1st day	0.00	1,700,000	0.00	11,000,000	
2nd day	0.54	1,500,000	5.76	60,000,000	
3rd day	0.90	2,700,000	10.44	101,000,000	770,000,000
4th day	1.71	2,000,000	12.15	105,000,000	690,000,000
6th day	4.68	15,000,000	Discontinued		

experimentally important point is the accumulation of cells in the foam. Foaming is a common difficulty in aeration experiments. Media containing peptone have a stable foam, and other media develop a stable foam by bacterial growth. The cell concentration in foam was always found to be much larger than in the liquid medium, and disregard of the foam may cause a great experimental error.

These aeration experiments explain very simply a phenomenon in multiplication of bacteria which has heretofore led to the assumption of specific, growth-inhibiting metabolic products. Eijkman (1905) working with *Escherichia coli* and independently Rahn (1906 and 1932) working with *Pseudomonas fluorescens* observed that cultures which had reached the maximal population, showed normal growth again if they were either filtered or heated, and reinoculated. Rahn could repeat this 6 times with the same culture, the multiplication becoming slower each time, but reaching nearly the same endpoint. In all, the total crop obtained by 6 successive cultures was about 10,000,000,000 cells per ml. while the growth of a normal culture ceased at about 1,000,000,000. He came to the same conclusion as Eijkman that the multiplication was stopped by a thermo-

labile compound secreted by the cells, an "autotoxin" which was destroyed by heat or adsorbed at the filter.

The present investigations show that the "normal" culture ceases to increase because bacteria die in the lower strata from lack of oxygen as rapidly as they multiply on the very surface. Filtration or heating kills or removes the bacteria, and multiplication begins anew after the sterilized liquid has dissolved new oxygen. The food concentration becomes gradually less, resulting in a slower multiplication rate, but the limiting factor for the final crop is to the very last the availability of oxygen.

VII. CONVECTION CURRENTS

In chapter III (J. Bact., **41,** p. 234), it had been shown that oxygen diffusion is very slow, and that saturation of liquids with dissolved oxygen is accomplished more readily by convection currents. These currents cannot be standardized experimentally; the only way to measure their influence is to compare liquid cultures which are subject to convection currents with either solid cultures, or with continuously agitated cultures which would represent the other extreme.

The best way to avoid convection currents without changing the liquid character of the culture is the addition of 0.1 to 0.2 per cent of agar. This simple means produces an excellent growth with clostridia, according to Spray (1933), while Rippel (1936) obtained a better nitrogen fixation by *Azotobacter*, and Virtanen (1936) a much better growth of *Aspergillus*. The effect with *Clostridium* is easily explained; the oxygen consumption by the medium is sufficient to prevent any oxygen from reaching the bottom of the testtube by diffusion (see Chapter III). The stimulation of the aerobic cultures is not so easily explained, and required some special experiments.

The general method employed in these experiments is shown in figure 2. Three large testtubes, each holding 50 ml. of broth, were sterilized and aerated after cooling by bubbling through them a current of air to which about 1 percent CO_2 had been added. When the broth was saturated with air, i.e., after about 30 minutes of aeration, the aeration tubes of two test tubes were pulled back above the surface of the broth so that the air was renewed, but the liquid not disturbed. All tubes were then inoculated and to one tube, enough melted 5 per cent agar was added to make the agar concentration 0.1 to 0.2 per cent. Thus, all three tubes had an equal inoculum and an equal oxygen concentration at the start. In one tube, the oxygen concentration was maintained at saturation by continuous aeration. In another, diffusion as well as convection currents were the means of replacing oxygen consumption, and in the third, convection had been prevented, and diffusion was the only means of oxygen replacement.

Three representatives of quite different groups were studied in this way. namely *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Streptococcus lactis*, With each species, two series of tests were made simultaneously, one with air and one with oxygen. In each case, carbon dioxide had been added to the gas used for aeration. The results are shown in table 3. In the analysis of this table, it must be considered that the initial oxygen concentration of all cultures

growing with air was the same, and that the oxygen concentration of all cultures treated with oxygen was also uniform, but 5 times as large as that of the set saturated with air. During the first 6 hours of growth, the oxygen concentration cannot have changed much, for the highest cell concentration reached was 600,000. Any differences during this time must therefore be due to other causes. After 24 hours, the oxygen at the bottom of all cultures must have been exhausted, excepting those with continuous aeration. Any differences at this time are primarily due to differences in oxygen supply. It must be further considered that the error in plating is considerably greater for the agar cultures

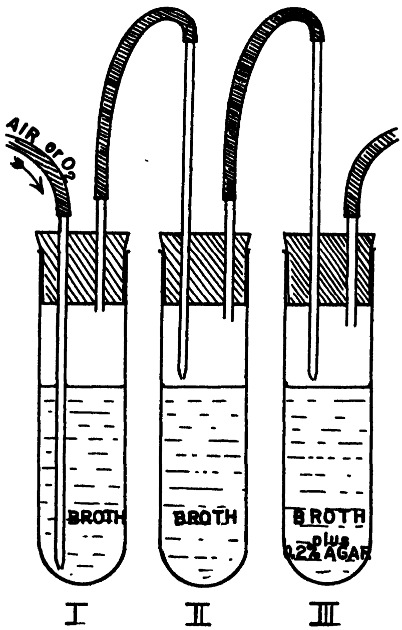

FIG. 2. METHOD USED IN AERATION EXPERIMENTS

All tubes are at first saturated with air or oxygen, then the aeration tube is withdrawn from the liquid in tubes II and III as shown.

than for liquid cultures, because the separation of individual cells will be less complete.

The first six hours of the tests for the three species are shown in figures 3, 4, and 5. They show that pure oxygen retarded the initial growth of *B. subtilis* and *S. lactis*, but not of *P. fluorescens*. This agrees with the observation of Berghaus (1907) that *Bacillus anthracis* was killed by 1.5 atmospheres of oxygen pressure in 24 hours, while *Pseudomonas pyocyanea* could tolerate 75 atmospheres. In most cases, the agar cultures are the first to come out of lag and multiply. As this happens before the oxygen concentration can have changed materially, it must be due to the absence of convection currents in the agar culture. This can be explained in the following way:

The transferred cells were old; they had ceased to grow, and their metabolism

TABLE 3

The effect of aeration by oxygen and by air on the multiplication of different bacteria

HOURS		OXYGEN			AIR		
		Continuous saturation	Convection + diffusion	Diffusion only	Continuous saturation	Convection + diffusion	Diffusion only
		Cells per ml. of *Bacillus subtilis*					
0		17,300	17,300	17,300	20,900	20,900	20,900
1		16,000	13,800	13,800	18,500	16,700	23,750
2		14,950	14,100	17,500	19,200	16,450	16,800
3		11,800	12,700	10,900	21,300	15,050	23,200
4		13,800	12,400	13,300	22,850	17,150	19,900
5		18,750	15,000		31,800	25,500	54,500
6		25,150	23,000	18,500	50,700	39,500	113,500
9.5	top	77,000	110,000	75,000	386,000	400,000	360,000
	bottom			245,000			1,015,000
24	top	371,000,000	63,000,000	51,000,000	251,000,000	38,000,000	15,000,000
	bottom			18,000,000			23,000,000
		Cells per ml. of *Pseudomonas fluorescens*					
0		41,100	35,700	37,000	20,600	16,700	14,800
1		37,800	34,700	37,400	18,700	15,500	17,300
2		40,100	34,100	38,900	15,300	14,700	18,000
3		44,200	36,700	31,000	17,900	17,100	17,100
4	top	52,700	44,000	45,100	21,700	17,600	24,700
	bottom			73,400			20,400
5		78,000	59,000	56,500	30,000	26,600	52,500
6	top	115,500	105,000	97,500	48,000	48,500	50,500
	bottom			171,000			37,200
9	top	506,500	570,000	1,485,000	310,500	241,500	530,000
	bottom			1,875,000			258,000
13	top	4,045,000	3,745,000	1,925,000	2,685,000	3,970,000	4,950,000
	bottom		6,000,000	2,440,000		4,905,000	840,000
25	top	1,620,000,000	179,000,000	412,000,000	430,000,000	140,000,000	39,000,000
	bottom		114,000,000	5,500,000		129,000,000	36,000,000
		Cells per ml. of *Streptococcus lactis*					
0		8,200	9,800	10,300	11,300	12,300	11,300
1		9,400	9,500	9,500	11,400	11,900	11,100
2		11,300	11,300	9,600	14,500	15,600	13,500
3		17,000	17,000	12,100	26,500	29,000	24,900
4	top	42,000	42,500	18,000	56,000	97,000	38,500
	bottom			37,500			67,000
5		116,500	136,500	70,000	220,000	281,000	291,000
6	top	309,000	371,000	160,000	660,000	1,085,000	770,000
	bottom			230,000			655,000
10	top	9,400,000	55,400,000	22,100,000	54,000,000	16,750,000	66,500,000
	bottom			3,050,000			85,000,000
12	top	74,000,000	86,000,000	382,000,000	398,000,000	544,000,000	623,000,000
	bottom			341,000,000			859,000,000
24	top	168,000,000	685,000,000	347,000,000	1,290,000,000	446,000,000	345,000,000
	bottom			700,000,000			445,000,000

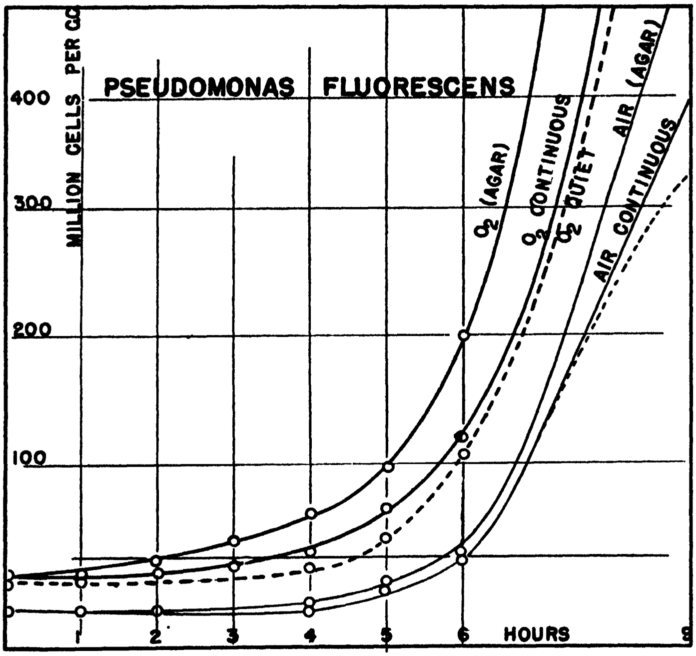

FIG. 3. THE FIRST 9 HOURS OF MULTIPLICATION OF PSEUDOMONAS FLUORESCENS IN AERATED AND QUIET CULTURES

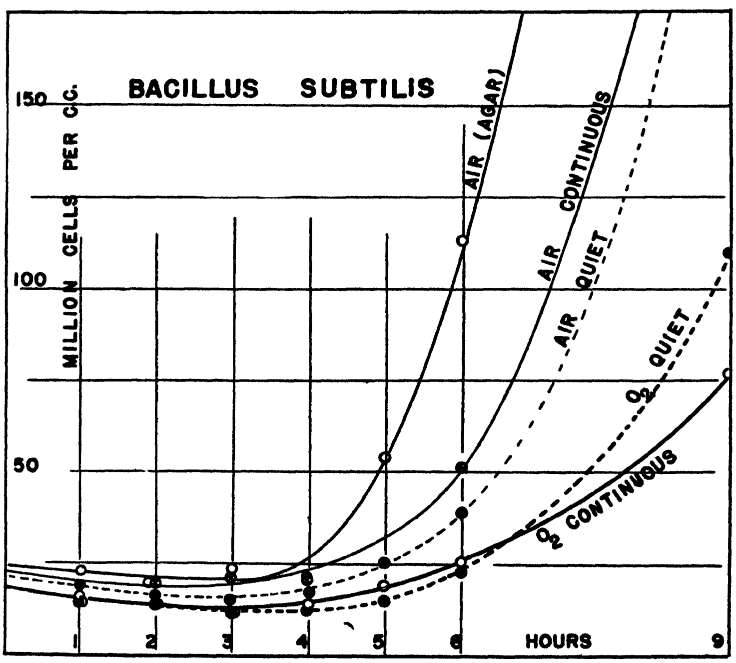

FIG. 4. THE FIRST 9 HOURS OF MULTIPLICATION OF BACILLUS SUBTILIS IN AERATED AND QUIET CULTURES

had become slow; the enzyme systems were no more fully active. In the new medium, the new environment makes its impact from all sides. The cell contents are not in equilibrium with the medium, and compounds begin to diffuse in and out. Among the incoming substances, oxygen is the most threatening. It would be welcome if the cells were prepared for it, but as the enzyme systems are still largely inactive, the oxygen begins to react with cell constituents. Perhaps the enzyme systems are prepared only to utilize the reserve materials of the cell, and new food does not diffuse as rapidly as oxygen. Doubtless, it takes some time before the enzymes are brought back to normal working condition. Up to this time, oxygen is threatening the vital parts of the cell, the native proteins and the molecules responsible for repair and for growth.

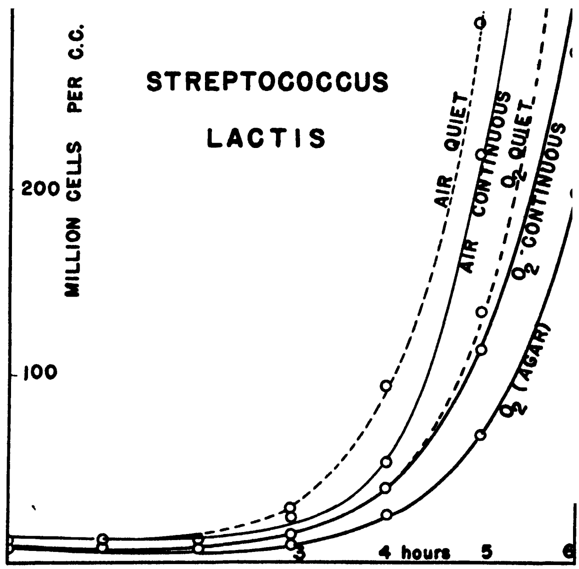

FIG. 5. THE FIRST 6 HOURS OF MULTIPLICATION OF STREPTOCOCCUS LACTIS IN AERATED AND QUIET CULTURES

If oxygen comes in too rapidly, it oxidizes vital parts irreparably, and causes death. The cell can cope only with a limited amount of oxygen. The rate of oxygen intake depends upon the concentration in the immediate surroundings of the cell. If the cell is imbedded in agar, the dissolved oxygen of the environment will be lowered by diffusion into the cell, and further oxygen diffusion into the cell will be much slower. In a liquid medium, the immediate neighborhood may be changed by convection currents; the oxygen-depleted medium around the cell may be replaced again and again by oxygen-saturated medium. The redox potential necessary for life may thus never be established in the cell or in its immediate surroundings, and the cell finally dies from exhaustion.

This theory has several general experiences in its favor. Aside from figures 3, and 4, and 5, there is figure 1 showing that aeration, i.e., agitation, retards development. The experiments have already been mentioned of Winslow,

Walker and Sutermeister who observed retardation by aeration, and even complete inhibition in a synthetic medium. Webster (1925) made *Bacterium lepisepticum* grow well and without lag at reduced oxygen pressure while it would not grow aerobically if the inoculum was small.

Another experience supporting this theory is the observation by Rahn (1906), Penfold (1914), Graham-Smith (1920), Ingraham (1933), and others that the lag phase becomes shorter when the inoculum becomes larger. It is easily seen

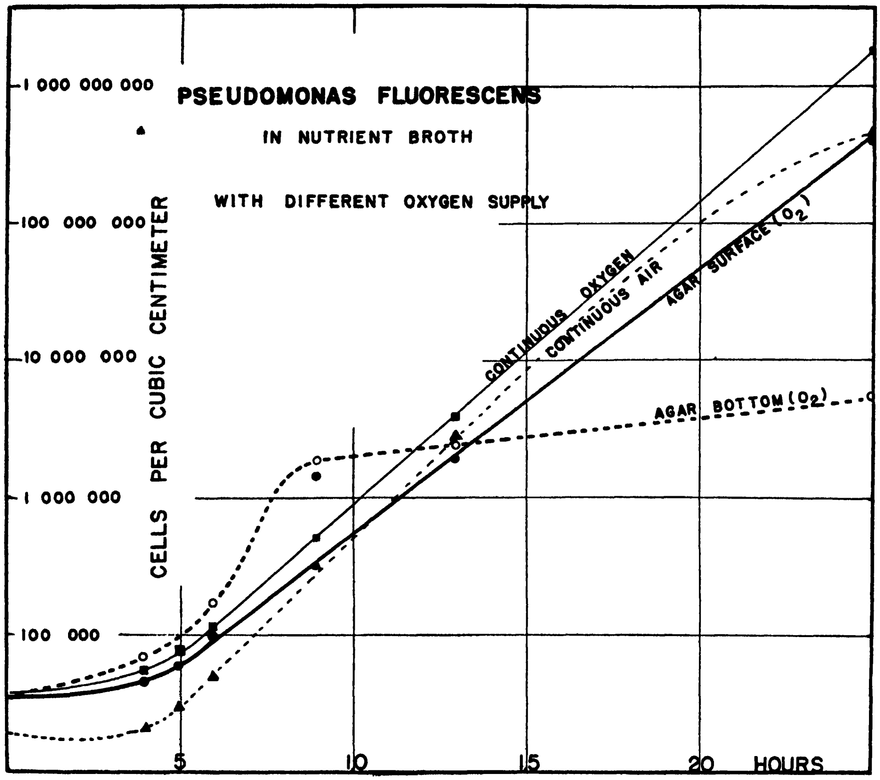

FIG. 6. MULTIPLICATION OF PSEUDOMONAS FLUORESCENS WITH DIFFERENT AMOUNTS OF OXYGEN SUPPLY

that more bacteria will be able to remove excess oxygen more rapidly, and thus establish optimal conditions for growth earlier.

The most striking example, however, is our general confidence in plate counts. We take it for granted that each single cell from a liquid culture will produce a colony in an agar plate. However, if we transfer the same liquid culture to a new medium, we transfer at least a loopful, and often 2 or 3 loopfuls, and if the culture is old, we may even transfer half a milliliter. This apparent contradiction in our everyday behavior is probably not due to thoughtlessness, but the result of empirical experience. It agrees with the above explanation.

The last 12 hours of the cultures of the two aerobic species agree with the state-

ments of the preceding chapters. As soon as the oxygen at the bottom of the liquid is exhausted, multiplication becomes slow or zero. Only from this time on do aerated cultures develop more rapidly then the quiet cultures. Figure 6 gives some of the logarithmic multiplication curves for *Pseudomonas.* It is worth noting that the oxygen-aerated culture still shows a constant growth rate after 25 hours when the cell concentration is more than a billion per ml. and the bacteria at the very surface of the corresponding agar tube also show no decline in the rate while the air-aerated cultures have begun to slow down. *Bacillus subtilis* also shows a higher final cell concentration in oxygen, but the difference is not great. Unfortunately, the fluctuation in the plate counts of top and bottom samples of the agar cultures are too great to permit any conclusions.

Quite different from the above two cultures is the *Streptococcus.* Oxygen retarded it more than air, and aeration by oxygen gave a surprisingly small final crop. Even the unaerated oxygen culture remained very low to the 12th hour. Since the cells consume hardly any oxygen, its concentration remains quite high for a long time. The larger number of cells in the air-aerated culture may be due to the effect of stirring.

SUMMARY

V. The rate of multiplication of most bacteria remains constant only for a few hours because the dissolved oxygen of the medium is soon exhausted, and from then on, the rate of multiplication is constant only at the very surface where oxygen from the air is available. Experiments permit the calculation that oxygen does not penetrate deeper than 2 mm. Below this thin surface stratum, facultative anaerobes multiply more slowly while strict aerobes do not multiply at all; usually, they decrease in number.

VI. Aeration does not necessarily increase the rate of multiplication, but it maintains it constant for a long time and thereby brings about a much larger bacterial crop. Aerated cultures may have 10 times as many bacteria as quietly standing cultures. With *Streptococcus lactis,* the number of cells is not greatly affected by aeration; pure oxygen decreases it.

VII. The delayed start of aerated cultures is explained by the assumption that a solution saturated with oxygen is harmful to resting cells whose enzyme mechanism is not working at the normal rate of multiplying cells. An old cell in a new environment establishes a zone of optimal conditions in its immediate neighborhood, probably at the expense of reserve compounds. If this is continually disturbed, e.g. by convection currents, the cell will finally die from exhaustion. The good growth on agar may be due merely to the prevention of convection currents.

REFERENCES

BERGHAUS, W. H. 1907 Ueber die Wirkung der Kohlensäure, des Sauerstoffs und des Wasserstoffs auf Bakterien bei verschiedenen Druckhöhen. Arch. Hyg., **62**, 172–189.

EIJKMAN, C. 1905 Ueber thermolabile Stoffwechselprodukte als die Ursache der natürlichen Wachstumshemmung der Bakterien. Zent. Bakt. Parasitenk., I, Orig., **37**, 436 and **41**, 367.

Graham-Smith, G. S. 1920 The behavior of bacteria in fluid cultures as indicated by daily estimates of the number of living organisms. J. Hyg. **19**, 133–145.

Howell, S. F., and Sumner, J. B. 1934 The specific effects of buffers upon urease activity. J. Biol. Chem. **104**, 619–626.

Ingraham, M. E. 1933 The bacteriostatic action of gentian violet and its dependence on the oxidation-reduction potential. J. Bact., **26**, 573–598.

Penfold, W. J. 1914 On the nature of bacterial lag. J. Hyg., **14**, 215–226.

Rahn, Otto 1906 Ueber den Einfluss der Stoffwechselprodukte auf das Wachstum der Bakterien. Zentr. Bakt. Parasitenk., II, **32**, 418–429.

Rahn, Otto 1932 Physiology of Bacteria. Baltimore, Blakiston, p. 235.

Rippel, A. 1936 Ueber die Wirkung von geringen Mengen Agar auf Wachstum und Stickstoffbindung von Azotobacter und auf andere mikrobiologische Vorgänge. Arch. Mikrobiol. **7**, 21–234.

Spray, R. S. 1933 A semi-solid medium for anaerobic cultivation. J. Bact., **25**, 38.

Virtanen, A. I. 1936 Bemerkungen zu der Arbeit von A. Rippel: "Ueber die Wirkung von geringen Mengen Agar auf Wachstum und Stickstoffbindung von Azotobacter und auf andere mikrobiologische Vorgänge. Arch. Mikrobiol., **7**, 488–489.

Webb, B. H. 1935 Color development in lactose solutions during heating. J. Dairy Sci., **18**, 81–96.

Webster, L. T. 1925 Biology of *Bact. lepisepticum.* J. Exptl. Med., **44**, 571–578.

Winslow, C.-E. A., Walker, H. H., and Sutermeister, M. 1932 The influence of aeration and of sodium chloride upon the growth curve of bacteria in various media. J. Bact., **24**, 185–208.

THE PRODUCTION OF ACTIVE RESTING CELLS OF STREPTOCOCCI

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Nearly all of the recent studies relating to the nutrition of microorganisms have been directed toward raising the largest possible cell crop in a medium of known chemical composition, without regard to the physiological activity of the cells harvested from the medium. The purpose of the present study has been to produce resting cell suspensions of streptococci with sufficient activity and stability to permit metabolic studies. In this first attempt to obtain highly active cells, knowledge of the chemical composition of the medium has been sacrificed. It should not be too much to expect, however, that it will be possible to raise, in media of known composition, cells of high physiological activity. A number of reports in the literature, including those of Farrell (1935) and Callow (1926) indicate the difficulties encountered in the preparation of physiologically active suspensions of streptococci.

METHODS

A typical strain of *Streptococcus mastitidis*, number 70b, (Lancefield group B) from the departmental stock culture collection was used in the experiments here reported. The general conclusions have, however, been confirmed with several strains of group B organisms, including two from human sources.

The Thunberg method using methylene blue as the hydrogen acceptor was employed as a criterion of cellular activity, or more precisely of dehydrogenase activity. This method was chosen with the hope of detecting mechanisms in the cells other than the pure lactic fermentation for which these organisms are known. The method also has the advantage of being rapid, and easy enough to handle a number of cultures when necessary.

The Thunberg experiments were run at 40°C. in the conventional tubes with the following quantities of reactants:

In side arm....................	1 ml. 1:4,000 methylene blue
In tube..........................	2 ml. M/15 phosphate buffer pH 7.2
	1 ml. M/20 substrate
	1 ml. cell suspension in M/30 phosphate buffer. (Cells 10 times the concentration in which they grew)

The tubes were allowed to come to the temperature of the bath and evacuated. After sufficient time for temperature equilibrium to be reached, the methylene blue was added from the side arm. The time required for 90 per cent reduction of the methylene blue present was recorded (Quastel and Whetham, 1924). In

most experiments this reduction time was relatively short, but agreement between duplicate tubes was well within 10 per cent.

Methylene blue as a hydrogen acceptor for work with streptococci has been objected to on the basis of its toxicity toward this group of organisms (Farrell, 1935). It is true that a very low concentration of methylene blue is toxic to the growth of streptococci, especially in broth. However, in these experiments, with resting cell suspensions of group B streptococci the toxicity of methylene blue was not an important factor. Figure 1 indicates that, with the cell concentration used, the toxicity of methylene blue is not apparent until a concentration greater than one part in twelve thousand is reached, as indicated by a linear relationship between reduction time and methylene blue concentration. One part of methylene blue in twenty thousand final concentration was selected as a satisfactory level. In earlier experiments one part of methylene blue in

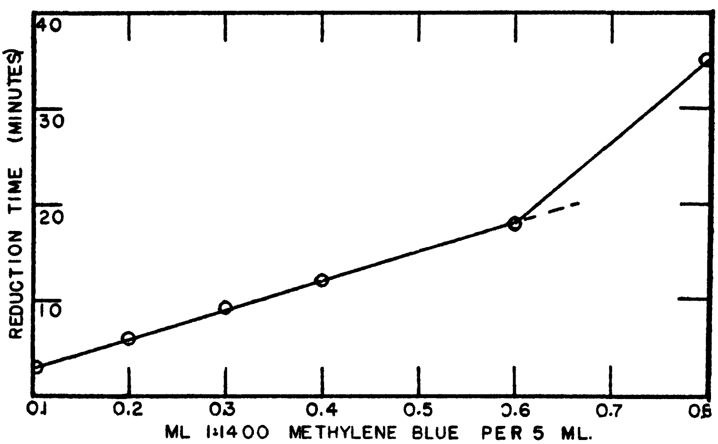

FIG. 1. EFFECT OF METHYLENE BLUE CONCENTRATION ON REDUCTION TIME

forty thousand was used and found to be satisfactory with more dilute cell sus pensions.

Glucose was used as substrate in determining conditions for the production of active cells. Its use could be criticised on the basis of the complexity of the reactions which it undergoes with these organisms. On the other hand, although glucose is readily metabolized by these organisms, adverse conditions frequently affect reactions with it more readily than with other substrates (Quastel and Wooldridge, 1927). In addition, glucose permits the study of adaptive enzymes for various carbohydrates.

The amount of growth was expressed in terms of bacterial nitrogen, as determined by the micro-kjeldahl method of Pregl (Niederl and Niederl, 1938). This method was selected as giving more accurate and reproducible results with these long chain-forming organisms than would the conventional plate count (Mueller, 1935). The method is not unduly tedious for the number of determinations required and gives the added advantage of yielding results rather promptly. No

attempt has been made to determine the proportion of dead cells in the suspensions.

Preliminary studies of factors involved in the preparation of cell suspensions included the age of the culture, incubation temperature, size of inoculum, and mode of washing the cells. For a given medium, cells of satisfactory activity could be obtained by growing at 37°C. and harvesting near the end of the logarithmic growth phase. Three washes with $\frac{1}{3}$ the growth volume of M/30 phosphate, pH 7.2, produced cells with a low endogenous rate of reduction of methylene blue without material decrease of the reduction rate in the presence of substrates. In all experiments recorded in this paper the time for methylene blue reduction in the absence of substrate was over two hours. In studies with substrates which had high reduction rates, less washing could be used without the endogenous respiration accounting for too great a percentage of the experimental values. Twelve to fifteen-hour cultures grown at 37°C. from a 0.1 per cent inoculum fulfilled the conditions described above and were therefore used in the following experiments.

After washing, the cells were suspended in $\frac{1}{10}$ their growth volume and 1 ml. of the suspensions was used in the Thunberg tubes, as described above. Thus, the values for bacterial nitrogen (figs. 2–5) are milligrams per 10 ml. of growth medium, equal to mgm. per ml. of cell suspension. Dilution of the cell suspensions gave the conventional type of curves as indicated by Quastel and Wooldridge, (1924) with *Escherichia coli* and by Farrell (1935) with streptococci. The conditions in these experiments were such that doubling the cell concentration approximately halved the reduction time, and halving the cell concentration approximately doubled the reduction time.

RESULTS

A number of media, as well as the effect of varying the amount of carbohydrate, were studied before it was found that cells of predictable activity could be grown in a medium composed of Bacto-tryptone, yeast extract, phosphate buffer, and a small amount of glucose. By varying the concentration in the growth medium of each of the above constituents while the others were held constant, the effect of each on the physiological activity and stability of the cell suspensions prepared from different media was determined.

Effect of tryptone

The effect of varying the concentration of tryptone from 0 to 2 per cent in a medium containing 1 per cent yeast extract, 0.5 per cent K_2HPO_4 and 0.1 per cent glucose is shown in figure 2. The quantity of bacterial nitrogen increased linearly from 0.13 mgm. per 10 ml. of medium in the absence of tryptone to 0.26 mgm. with 2 per cent tryptone, or a twofold increase. The rate of reduction of methylene blue by resting cell suspensions was three times as great in a medium containing 1 per cent tryptone as in a medium without tryptone. The increase in quantity of cells, about 50 per cent in the presence of 1 per cent tryptone, is not sufficient to account for the increased activity. A further increase

in tryptone from 1 to 2 per cent gave approximately the same increase in growth as from 0 to 1 per cent, with only a slight increase in the methylene blue reduction rate. In subsequent experiments 1 per cent tryptone was used as a satisfactory concentration.

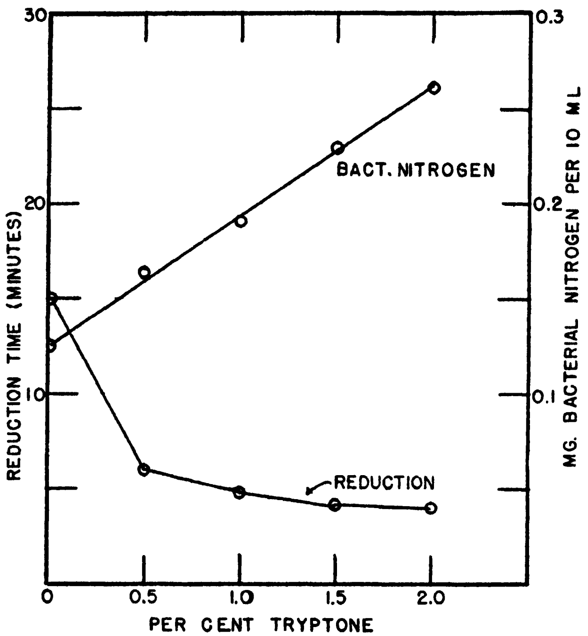

FIG. 2. EFFECT OF TRYPTONE CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP AND RATE OF METHYLENE BLUE REDUCTION BY THE CELL SUSPENSIONS HARVESTED FROM THE MEDIUM

Effect of yeast extract

The effect of varying the yeast extract concentration from 0 to 2 per cent in a medium containing one per cent tryptone, 0.5 per cent K_2HPO_4 and 0.1 per cent glucose is shown in figure 3. Only a small increase in bacterial nitrogen occurred with the increase in the yeast extract concentration, but a very marked increase in the reduction rate did occur. The addition of 0.2 per cent yeast extract, to the growth medium, increased the methylene blue reduction rate of the cell suspensions harvested from the medium by 400 per cent, whereas the growth was increased by less than 10 per cent. Further increase in the yeast extract concentration from 0.2 to 1 per cent approximately doubled the reduction rate, while the growth increased by about 15 per cent. Further additions of yeast extract, up to 2 per cent, increased neither the quantity of growth nor the activity of the cells harvested from the medium.

Upon storage of the cell suspensions in the refrigerator those cells harvested from media containing yeast extract were more stable than those from media which contained only tryptone, phosphate, and glucose (table 1). Cells har-

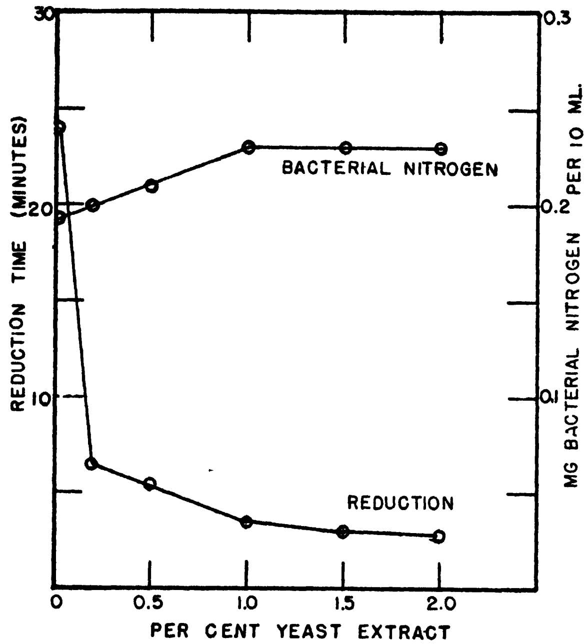

FIG. 3. EFFECT OF YEAST EXTRACT CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP AND RATE OF METHYLENE BLUE REDUCTION BY CELL SUSPENSIONS HARVESTED FROM THE MEDIUM

TABLE 1

Effect of growth in yeast extract on the stability of cells during storage

CONC. OF YEAST EX. IN MEDIUM	MINUTES FOR 90 PER CENT REDUCTION OF 1:20,000 METHYLENE BLUE		ACTIVITY RETAINED
	Immediately after centrifuging	After 24 hours at 8°C.	
Twelve-hour cells			
per cent			*per cent*
0	24.00	>60	<40
0.2	6.00	8.8	68
0.5	5.25	8.0	65
1.0	3.50	5.5	65
1.5	3.10	3.4	91
2.0	2.80	3.3	85
Twenty-four-hour cells			
0	28.0	>60	<45
0.2	10.5	23	45
0.5	8.0	19	42
1.0	5.5	10.5	52
1.5	3.6	7.0	50
2.0	3.10	6.0	50

vested from 12-hour cultures, in addition to having greater activity, were more stable than those harvested from 24-hour cultures. Therefore 12- to 15-hour cultures were used.

A concentration of 1 per cent yeast extract was considered satisfactory for most work, but occasionally 1.5 per cent was used when cells were to be stored. No attempt has been made in this work to ascertain which constituent or constituents of the yeast extract were responsible for this marked effect.

Effect of buffer

The buffer concentration in a medium containing 1 per cent each of tryptone and yeast extract and 0.1 per cent glucose was varied from 0–0.1 molar (fig. 4)

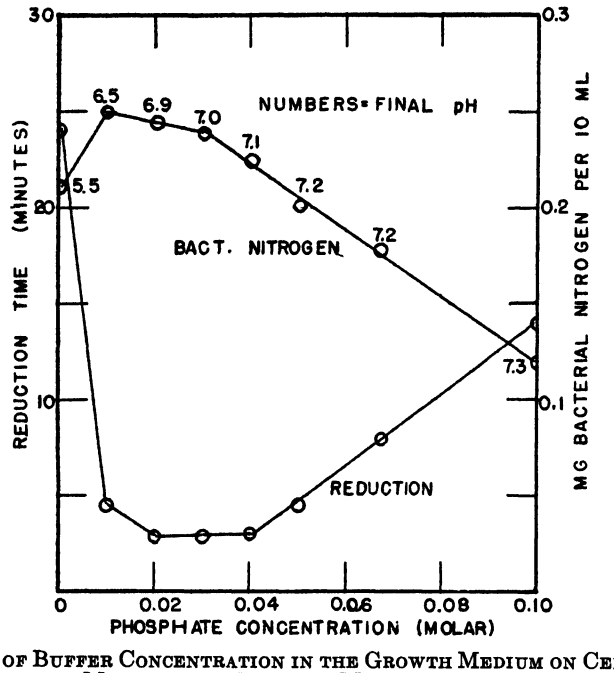

FIG. 4. EFFECT OF BUFFER CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP, FINAL pH IN THE MEDIUM, AND RATE OF METHYLENE BLUE REDUCTION BY CELLS HARVESTED FROM THE MEDIUM

Small concentrations of buffer increased the quantity of growth slightly. This is compatible with the work of Hewitt (1932) which indicates a stimulation in the rate of the lactic fermentation on the addition of phosphate. On the other hand, the stimulatory effect may be due to the buffering capacity which would keep the reaction at a favorable level for growth over a longer period of time. Concentrations of buffer above 0.04 molar showed an inhibitory effect on the growth of these group B streptococci. The methylene-blue reduction rate was increased markedly up to a concentration of 0.02 molar phosphate. Although the concentration of phosphate ions over this range may be of importance in determining the cellular activity, the increased buffering action must not be over-

looked. (The effect of the final pH in the growth medium will be discussed with the effect of the sugar content of the medium.) In the range from 0.02 to 0.04 molar phosphate the amount of growth and the reduction rate, as well as the final pH of the medium, (indicated by numbers above bacterial nitrogen curve) were approximately constant. Above 0.04 molar phosphate the cellular activity, as well as the amount of growth, decreased. Therefore, a phosphate concentration of 0.03 molar (0.5 per cent) was used for growth of group B organisms.

Effect of glucose

By varying the glucose concentration in a medium containing 1 per cent each of tryptone and yeast extract and 0.05 per cent K_2HPO_4, a marked effect of the

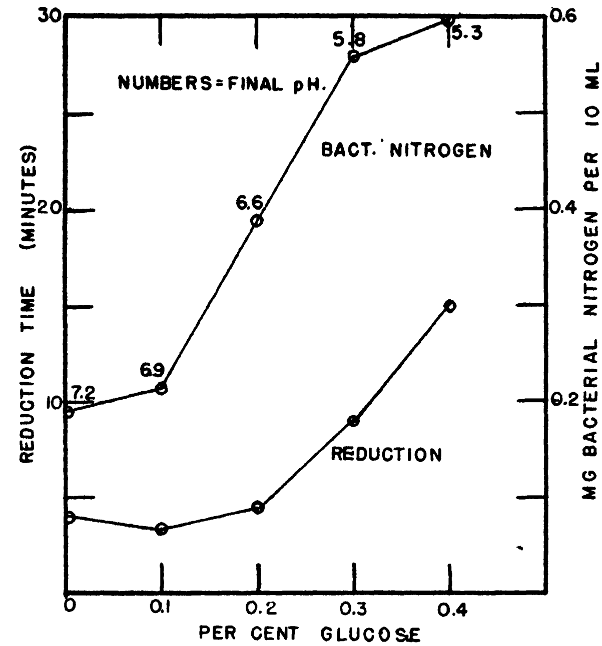

FIG. 5. EFFECT OF GLUCOSE CONCENTRATION IN THE GROWTH MEDIUM ON CELL CROP, FINAL pH IN THE MEDIUM, AND RATE OF METHYLENE BLUE REDUCTION BY THE CELLS HARVESTED FROM THE MEDIUM

final pH in the growth medium upon the activity and stability of the cells appears (fig. 5). The numbers just above the bacterial nitrogen curve indicate the pH in the growth medium from which the cells were harvested. Cells grown in a medium which became more acid than pH 6.8 had less activity than those grown in a medium which was not allowed to reach this acidity. Very great decrease in the cell activity occurred when the final pH in the growth medium fell below 6.5. As can be seen from figure 5, there is a marked decrease in the methylene blue reduction rate in spite of a great increase in the cell nitrogen. With the higher levels of glucose the endogenous respiration was increased somewhat.

DISCUSSION

The Thunberg method is applicable to the streptococci, which have strong dehydrogenase activity when the cells are properly prepared. The toxicity of methylene blue for these organisms is not so great in resting suspensions as in growing cultures. The more exacting nutritional requirements, the generally low level of synthetic powers, and the lack of an adequate aerobic mechanism among these organisms necessitate more exacting conditions for the preparation of resting cells. For example, strong aeration, as suggested by Quastel and Whetham (1925) for the removal of reducing material from suspensions of *E. coli* in the production of resting cells has been used by Farrell (1935) and Katagiri and Kitahara (1938) with streptococci and lactobacilli. The aeration, satisfactory in the preparation of resting cell suspensions of *E. coli*, is not only unnecessary but deleterious when applied to these lactic acid types. With streptococci the age of the culture is of more importance than with some types of bacteria.

The necessity of accessory factors or amino acids for the production of active cells is indicated by the stimulatory effect of higher levels of yeast extract and tryptone.

The fact that it is possible to raise resting cells of increased activity, that is, greater rate of methylene-blue reduction without increased growth, in a medium rich in yeast extract, suggests the possibility of a higher level of accessory substances per cell. Additional evidence of this possibility is offered by the observation that above a certain level, when more cells are raised per volume of medium, the activity is related to the volume of medium from which the cells were harvested and not to the quantity of cells.

The decrease in cellular activity when the final pH in the growth medium falls serves also to indicate the sensitivity of these organisms. This result recalls the observation of Lwoff and Lwoff (1937) on the sensitivity of *Haemophilus influenzae* to lowered pH in the growth medium.

It is our opinion that a number of observations on the absence of dehydrogenase activity in streptococci, and variability in activity of cell suspensions, may be traced to variation in one or another of these factors.

The use of methylene blue as a hydrogen acceptor is a method of setting up an artificial system the reaction rate of which may not be an accurate index to the metabolism of the organism. For example, it has not been shown that the conditions outlined for the production of cells with a high dehydrogenase activity, using methylene blue as the hydrogen acceptor, are necessarily the best conditions for the lactic fermentation. The criticisms offered by Barron and Jacobs (1938) as to the possibility of not observing certain dismutation reactions by this method has not been considered. This would not, however, invalidate positive results obtained by the method.

It is possible to produce streptococcus cells of high metabolic activity and low endogenous metabolism by growing them in a medium containing a high concentration of nitrogenous constituents and accessory factors and a low concen-

tration of carbohydrate, or energy source. Under these conditions the cells will cease to multiply for lack of energy source while they are in a neutral medium of high nutritional quality. Because their energy-supplying mechanism is depleted, the cells will have a low endogenous metabolism. This is more easily accomplished with streptococci than with a number of other organisms because fewer substances serve as satisfactory energy sources.

SUMMARY

Resting cell suspensions of streptococci of high physiological activity, low endogenous respiration, and fair stability have been produced. This has been accomplished by growing them in a well-buffered medium, rich in nitrogen and accessory factors, and low in carbohydrate, followed by washing in neutral phosphate buffer. It has been shown that streptococci grown under these conditions have, in resting suspensions, strong dehydrogenase activity.

The cells should be harvested near the end of the logarithmic growth phase, from a medium that is not allowed to become more acid than pH 6.8.

Methylene blue is not toxic to resting cell suspensions of group B streptococci at a concentration of 1 part in 20,000. Therefore, this concentration, or less, of methylene blue is satisfactory as a hydrogen acceptor in dehydrogenase studies.

REFERENCES

Barron, E. S. G., and Jacobs, H. R. 1938 Oxidations produced by hemolytic streptococci. J. Bact., **36**, 433–449.

Callow, A. B. 1924 The oxygen uptake of bacteria. Biochem. J., **18**, 507–518.

Farrell, M. A. 1935 Studies on the respiratory mechanisms of the streptococci. J. Bact., **29**, 411–435.

Hewitt, L. F. 1932 Bacterial metabolism. 2. Glucose breakdown by pneumococcus variants and the effect of phosphate thereon. Biochem. J., **26**, 464–471.

Katagiri, H., and Kitahara, K. 1938 The lactic dehydrogenase of lactic acid bacteria. Biochem. J., **32**, 1654–1657.

Lwoff, A., and Lwoff, M. 1937 Studies on codehydrogenases. II. Physiological function of growth factor "V." Proc. Roy, Soc. (London), **122B**, 360–373.

Mueller, J. H. 1935 Studies on the cultural requirements of bacteria. IV. Quantitative estimation of bacterial growth. J. Bact., **29**, 383–387.

Niederl, J. B., and Niederl, V. 1938 Micromethods of quantitative organic elementary analysis. New York, John Wiley & Sons.

Quastel, J. H., and Whetham, M. D. 1924 The equilibria existing between succinic, fumaric and malic acids in the presence of resting bacteria. Biochem. J., **18**, 519–534.

Quastel, J. H., and Whetham, M. D. 1925 Dehydrogenations produced by resting bacteria I. Biochem. J., **19**, 520–531.

Quastel, J. H., and Wooldridge, W. R. 1927 The effects of chemical and physical changes in environment on resting bacteria. Biochem. J., **21**, 148–168.

NUTRILITE REQUIREMENTS OF OSMOPHILIC YEASTS[1]

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INTRODUCTION

Osmophilic yeasts comprise an economically important group of organisms through their ability to grow in concentrations of sugar which suppress "ordinary" yeasts and thereby cause spoilage of various food products of high sugar content by alcoholic fermentation. Various species causing spoilage of honey have been described by Nussbaumer (1910), Richter (1912), Marvin (1928), Fabian and Quinet (1928), Lochhead and Heron (1929) and Sacchetti (1932a). Sugar-tolerant yeasts have been isolated from cane syrup by Owen (1913) and Hall, James and Nelson (1937), from maple syrup by Fabian and Hall (1933), from concentrated grape must by Kroemer and Krumbholz (1931) and Sacchetti (1932b), from cream fondant candies by Shutt (1925) and Church, Paine and Hamilton (1927), from dried fruits by Mrak and Baker (1940), while in this laboratory similar types have been found as the cause of spoilage of orange marmalade, molasses, maple butter and candies.

Though species of *Saccharomyces*, *Schizosaccharomyces*, *Torula* and *Mycotorula* have been found capable of growing in solutions of high sugar concentration, members of the genus *Zygosaccharomyces* are particularly prominent as sugar-tolerant forms. Not only are these yeasts the most commonly encountered osmophilic types, but certain species of *Zygosaccharomyces*, even when outnumbered by other yeasts, may develop and play the chief part in fermentation (Lochhead and Farrell, 1931).

For the proper nutrition of the common yeast, *Saccharomyces cerevisiae*, it is now recognized that in addition to a suitable supply of salts and sugar with adequate nitrogen source, accessory growth-promoting nutrilites are required. Such growth factors have usually been referred to collectively as "bios" or as contributing to the so-called "bios effect" first demonstrated by Wildiers (1901).

The multiple nature of "bios" came to be recognized chiefly through the work of Miller and associates (*cf.* Eastcott, 1941). Following the discovery by Eastcott (1928) of inositol as the first pure substance with "bios" activity, yeast-growth-promoting properties of other compounds have been demonstrated—thiamin by Williams and Roehm (1930); biotin by Kögl and Tonnis (1936); pantothenic acid by Williams *et al.* (1933) as well as its cleavage product, β-alanine by Williams and Rohrmann (1936); and pyridoxin (vitamin B_6) by Schultz, Atkin and Frey (1939) and Eakin and Williams (1939).

More recently Williams, Eakin and Snell (1940) have shown the relationship

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of inositol, biotin, pantothenic acid, thiamin and pyridoxin to the growth of strains of *Saccharomyces cerevisiae*. Such findings have not only helped to confirm Miller's work on the complementary effect of "bios" fractions, but have shown that the effect of "bios" is due essentially to a mixture of substances now recognized as vitamins, a relationship first postulated by Williams (1919).

Growth-factor requirements for osmophilic yeasts were first indicated by Farrell and Lochhead (1931) who found *Zygosaccharomyces mellis* to respond to the addition of at least two substances present in complementary fractions derived from honey. Neither substance was identical with inositol, when the yeast was tested with Bios I and Bios II of Miller. Farrell (1935) later found Bios IIA and Bios IIB necessary for growth of the same species which, unlike Wildier's yeast, gave little response upon addition of Bios I.

In view of the lack of information as to growth requirements of sugar-tolerant yeasts as a group, and the availability of crystalline substances with known "bios" effect, the experiments here reported were planned to note the effect of the important yeast nutrilites (*cf.* Williams, 1941) upon the growth of 23 strains of the genus *Zygosaccharomyces*. These represented 18 species, all but six of which were isolated in this laboratory, the sources being indicated in table 1.

METHODS

The yeasts were cultured in 200-ml. Erlenmeyer flasks containing 30 ml. of basal salts-sugar solution, with addenda as required to give a total volume of 40 ml. The basal solution was prepared to give a final concentration as follows: KH_2PO_4, 0.2 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; $(NH_4)_2SO_4$, 0.3 per cent; $CaCl_2$, 0.025 per cent and glucose, 40 per cent. Nutrilites were added as required sufficient to give a final concentration per ml. as follows: inositol, 50 μg; pantothenic acid (calcium pantothenate Merck), 0.1 μg; biotin (S.M.A. Corporation), 0.0001 μg; thiamin (S.M.A. Corp.), 0.1 μg and pyridoxin (S.M.A. Corp.), 0.2 μg. Beta-alanine, when used in place of pantothenic acid, was added at the same concentration. The flasks were autoclaved at 15 lb. pressure for 10 minutes. This treatment minimized caramelization of sugar and proved adequate with the concentration of glucose used.

The yeast suspensions for inoculation were prepared from 4- to 8-day cultures on glucose-honey-yeast agar of 40 per cent sugar concentration. Cells were washed three times in 10 per cent glucose solution and finally suspended in the basal medium. All suspensions were standardized to a turbidity giving 40 per cent light transmission of the clear basal solution. Flasks were inoculated, in duplicate, with 1 drop (0.05 ml.) of standard suspension.

Cultures were incubated at 28°C. Since osmophilic yeasts differ from *Saccharomyces cerevisiae* in being economically important as spoilage organisms rather than as useful agents of industrial fermentation, and since they develop more slowly, growth after longer periods of incubation may be considered of more importance than in the case of the latter yeast. Incubation was continued for eight days during which time flasks were shaken daily. Measurements of growth were made usually at two-day intervals by means of a Luxtrol photo-

electric turbidimeter. In addition to the variations in growth factor addenda being studied, all series of flasks included inoculated controls of the basal medium,

TABLE 1

Effect of omission of single nutrilites on growth of osmophilic yeasts in 40 per cent glucose solution

Culture			Percentage light transmission (4 days, 28°C.) (uninoculated control = 100)							
				With addition of growth factors						
No.	Name	Source	Control salts-sugar	All 5 growth factors	No inositol	No pantothenic acid	No biotin	No thiamin	No pyridoxin	All 5[1], with β-alanine
	Group A									
H3	*Zygosaccharmoyces sp.*	Maple butter	97.3	79.4	78.3	82.6	97.2	75.8	78.4	78.0
S3	*Zygosaccharomyces sp.*	Maple equipment	97.3	66.6	67.1	71.4	99.4	64.5	67.1	66.9
S5	*Zygosaccharomyces sp.*	Maple equipment	97.4	71.4	75.1	74.3	99.4	68.6	74.0	73.8
C3	*Zygosaccharomyces sp.*	"Exploded" candy	98.1	76.3	76.3	77.9	99.9	72.8	75.7	74.5
Z1	*Z. variabilis*	(K. and K.)[2] grapes	97.6	79.4	81.3	78.8	96.6	77.0	79.1	78.2
Z2	*Z. felsineus*	(S)[3] conc. wine must	97.3	68.6	68.2	73.1	95.6	69.4	71.3	72.2
Z5	*Z. nadsonii*	N.C.T.C.[4]	98.9	74.5	74.7	78.2	97.0	70.4	71.2	72.4
	Group B									
139	*Z. rugosus*	Normal honey	98.1	68 0	66.4	88.4	97.9	69.3	70.4	
Ex4	*Z. rugosus*	Honey tank	99.4	64.1	61.2	92.1	99.8	63.1	63.8	63.4
S6	*Zygosaccharomyces sp.*	Maple equipment	98.7	77.3	76.6	82.9	100.1	76.5	77.5	78.0
58	*Z. barkeri*	Normal honey	96.1	71.0	70.6	86.2	98.9	72.7	72.0	67.0
Z4	*Z. barkeri*	N.C.T.C.	98 3	71.0	74.4	90.2	99.2	76.8	75.0	74.1
A2	*Zygosaccharomyces sp.*	"Exploded" candy	100.0	70.2	68.6	80.1	100.0	67.5	71.7	72.2
M1	*Z. richteri*	Fermented honey	99.1	67.7	68.0	84.6	97.4	74.2	71.3	
Z3	*Z. amoeboidus*	(K. and K.) fruit syrup	99.7	63.7	64.7	73.4	100 5	56.9	61.5	61.7
427	*Z. acidifaciens*	(N)[5] acid wine	99.7	70.9	71.7	80.2	99.5	74.5	70.3	
	Group C									
9	*Z. japonicus*	Normal honey	96.9	66.9	68.4	96.2	98.3	65.5	66.6	61.3
D1	*Z. mellis*	Fermented honey	100.0	76.7	77.2	98.9	97.9	73.7	73.7	
J7	*Z. nussbaumeri*	Fermented honey	99.2	65.2	73.1	93.7	96.7	73.2	55.6	
E6	*Zygosaccharomyces sp.*	Fermented honey	99.4	59.9	67.1	99.2	99.7	62.0	55.9	64.8
N4	*Z. nectarophilus*	Floral nectar	99.5	79.7	93.4	98.5	97.9	82.1	83.3	
155Y	*Z. nectarophilus*	Normal honey	98.5	72.7	84.6	93.1	93.4	80.5	71.5	70.3
S3B2	*Z. nectarophilus*	Soil	99.4	63.5	67.9	98.2	101.0	63.6	63.0	63.4

[1] Beta-alanine in place of pantothenic acid.
[2] Kroemer and Krumbholz.
[3] Sacchetti.
[4] National Collection of Type Cultures, London.
[5] W. J. Nickerson, Jr.

both alone and with the combined growth factors, as well as of the uninoculated basal medium.

EXPERIMENTAL RESULTS

Effect of omission of single nutrilites

The effect of omitting single growth factors from the combination of five upon the growth of 23 yeast strains is shown in table 1, giving readings after 4 days at 28°C. At this stage of development the ability to synthesize the missing nutrilite, where such exists, should become manifest, so that it is possible to evaluate the importance of each as an essential factor for more prolonged growth.

Biotin is seen to be the most important nutrilite, being essential to the growth of all strains of *Zygosaccharomyces* studied. In its absence, growth of the organisms is little or no better than that in pure salts-sugar solution.

With respect to their need for pantothenic acid, the yeasts could be divided into three groups, depending upon whether this nutrilite was essential, stimulatory, or relatively unimportant. The organisms in group A are evidently able to synthesize this factor to meet their requirements, since its omission causes little or no diminution of growth. The yeasts of group B, though able to grow in the absence of pantothenic acid, develop definitely better when it is supplied, and thus comprise a group intermediate between group A and group C. This latter group includes yeasts for which pantothenic acid is most essential, the omission of this nutrilite resulting in growth approximating that in the salts-sugar control after four days. This points to a lack of ability to synthesize this factor under the test conditions. Table 1 also shows that β-alanine, where it was tested, was able to replace pantothenic acid in providing the combined growth factor effect.

The omission of inositol was, in most cases, without effect on growth. In the case of five strains in Group C, representing three species, inositol was found necessary for optimum growth, being particularly important for *Zygosaccharomyces nectarophilus*. In no case, however, did the lack of this factor prevent growth as compared with the control, suggesting a capacity either for replacement by other factors, or for synthesis of inositol in varying degree.

Under the experimental conditions neither thiamin nor pyridoxin was important for growth of most of the yeasts. With some cultures omission of thiamin resulted in slightly depressed growth, most noticeable in the case of *Zygosaccharomyces richteri*, *Z. acidifaciens*, *Z. nussbaumeri* and one strain of *Z. nectarophilus*. This effect was less pronounced as growth progressed, suggesting a capacity for synthesis of this factor. With other yeasts, especially *Z. amoeboidus*, somewhat better growth was noted without thiamin, pointing to a slightly depressing effect of this factor at the concentration used. Omission of pyridoxin caused little or no difference in the growth of osmophilic yeasts, the most pronounced effect being with *Z. nussbaumeri*, on which this nutrilite appeared to have a somewhat depressing action.

Supplementary effects of nutrilites

With biotin, pantothenic acid (or β-alanine) and inositol seen as the important nutrilites for osmophilic yeasts, further studies were made of individual and

combined effects in the light of the data in table 1. For this series representative yeasts from each of groups A, B and C were chosen, *Zygosaccharomyces variabilis* (Z1), *Z. rugosus* (139), and *Z. nectarophilus* (N4). In addition to the flasks containing the basal and combined growth factor controls as well as those in which individual nutrilites were omitted, the series included the modifications of addenda outlined in table 2.

The effect of single and combined nutrilites on the three types of yeast is illustrated in table 2, giving the readings after six days. However, the salient features are shown graphically in figures 1–4, which indicate more clearly differences between the species with respect to their requirements of biotin, pantothenic acid and inositol during the course of growth for 8 days.

TABLE 2

Effect of single and combined nutrilites on three yeasts of different growth requirements

SALTS-SUGAR (40 PER CENT GLUCOSE) PLUS ADDENDA AS BELOW	PERCENTAGE LIGHT TRANSMISSION (6 DAYS 28°C.)		
	Z. variabilis (group A)	*Z. rugosus* (group B)	*Z. nectarophilus* (group C)
(1) Nil	99.4	96.9	97.7
(2) Combined nutrilites	79.2	63.4	62.8
(3) No inositol	78.7	62.4	79.2
(4) No pantothenic acid	78.8	74.7	95.7
(5) No biotin	97.7	96.3	96.1
(6) No thiamin	74.7	64.6	63.3
(7) No pyridoxin	78.8	64.7	64.0
(8) Biotin alone	85.7	75.8	98.7
(9) Pantothenic acid alone	97.9	94.8	97.4
(10) Biotin + inositol	81.9	68.3	98.7
(11) Biotin + pantothenic acid	75.1	64.5	85.7
(12) Biotin + thiamin	82.4	71.8	98.9
(13) Biotin + pyridoxin	83.7	70.7	99.2
(14) Biotin + pantothenic acid + inositol	78.8	62.1	62.0
(15) Biotin + pantothenic acid + thiamin	83.1	63.3	79.1
(16) Biotin + pantothenic acid + pyridoxin	79.1	63.7	81.6

Though all three species are alike in requiring biotin for growth, they vary markedly in their capacity for growth without pantothenic acid. In contrast to *Z. nectarophilus*, for which the latter is essential, *Z. variabilis* is able to synthesize this factor in sufficient amounts for abundant growth, even though development is somewhat retarded at first. For *Z. variabilis* and *Z. rugosus* biotin alone produces definite though sub-maximal growth; together with pantothenic acid, which alone is ineffective, it gives growth equal to the combined nutrilite effect. With the former yeast there is evidence that in the presence of biotin, the other nutrilites can replace pantothenic acid in promoting growth, an effect not noticeable with *Z. rugosus*.

With *Z. nectarophilus* biotin or pantothenic acid alone is ineffective, though

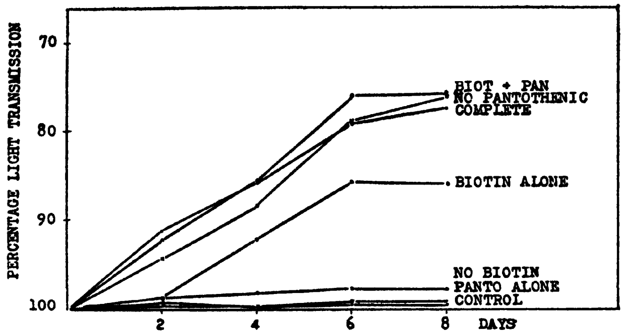

FIG. 1. GROWTH OF ZYGOSACCHAROMYCES VARIABILIS K. AND K. IN 40 PER CENT GLUCOSE SOLUTION

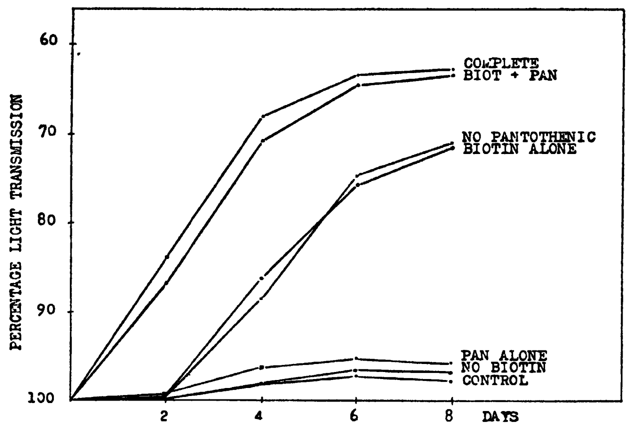

FIG. 2. GROWTH OF ZYGOSACCHAROMYCES RUGOSUS L. AND F. IN 40 PER CENT GLUCOSE SOLUTION

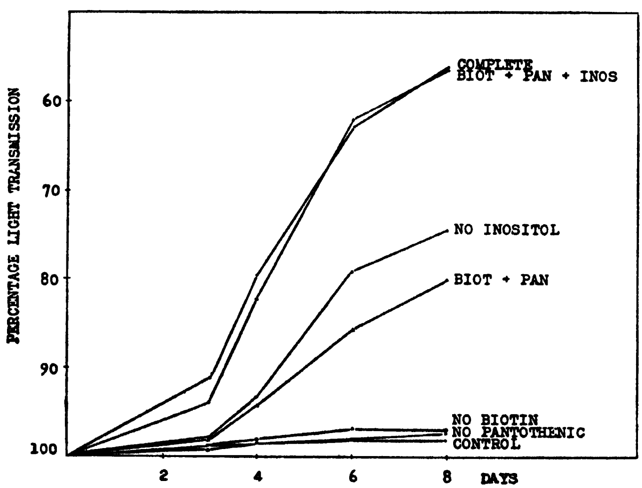

FIG. 3. GROWTH IN ZYGOSACCHAROMYCES NECTAROPHILUS L. AND F. IN 40 PER CENT GLUCOSE SOLUTION

together they promote sub-maximal growth. Upon the addition of inositol, a nutrilite which is non-essential for the other species, the combined growth factor effect is obtained, though growth is somewhat retarded at first. This suggests some early influence of thiamin and pyridoxin, later synthesized in sufficient amounts for optimum growth. A special series, in which this yeast was tested n a nutrient solution of 60 per cent glucose, confirmed the findings with the 40 per cent glucose medium (fig. 4). The osmophilic qualities of the yeast were shown by the fact that in an otherwise similar series with 10 per cent glucose no growth of the organism was obtained.

In its nutrilite requirements *Z. nectarophilus* resembles the Toronto strain of *Saccharomyces cerevisiae* which requires, in addition to Bios I (inositol), Bios IIA and Bios IIB, the growth-promoting effects of which are now coming to be regarded as similar to those exerted respectively by pantothenic acid (or β-alanine) and biotin.

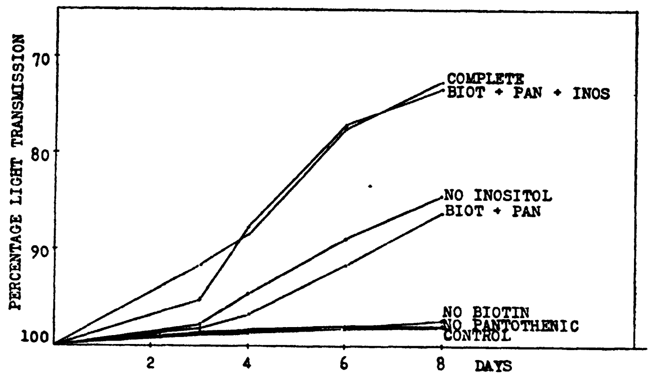

FIG. 4. GROWTH IN ZYGOSACCHAROMYCES NECTAROPHILUS L. AND F. IN 60 PER CENT GLUCOSE SOLUTION

DISCUSSION

Recently biochemical classification of *Saccharomyces* strains, based on growth factor tests, have been described by Rainbow (1939) and Schultz, Atkin and Frey (1940). The former classified the yeasts according to their reaction towards "bios" fractions. The latter based their classification on the growth response in a basal medium (which included inositol, β-alanine and Bios IIB), and in the same medium after the addition respectively, of thiamin and thiamin plus pyridoxin. Our experiments included a study of this point but under the conditions of our tests, carried out as they were with yeasts of a different genus, we were unable to observe any such clear-cut differentiation into types on the basis of response to thiamin and pyridoxin. A number of our strains might be regarded as belonging to their type B (growth depressed somewhat by thiamin and normal with thiamin and pyridoxin, as compared with growth in presence of inositol, pantothenic acid and biotin), though none would be considered as belonging to their Types A or C.

It was of interest to note that strains of *Zygosaccharomyces* which had been previously considered the same species on the basis of morphological and fermentation tests agreed with respect to their group classification on the basis of their pantothenic acid requirements (table 1). Thus, cultures S3 and S5 were regarded as the same species in Group A, while Group B contains two strains each of *Z. rugosus* and *Z. barkeri*, and Group C three strains of *Z. nectarophilus*. Since most of the strains had been isolated more than ten years previous to the present tests, it appears that nutrilite requirements of osmophilic yeasts represent, to some degree at least, a specific characteristic which may aid in their classification.

SUMMARY

Twenty-three strains of osmophilic yeasts of the genus *Zygosaccharomyces* and representing 18 species, were studied with respect to their requirements of inositol, biotin, pantothenic acid, thiamin and pyridoxin, added to a basal solution of inorganic salts and 40 per cent glucose.

Biotin was essential to the growth of all strains. On the basis of their need for pantothenic acid, the yeasts could be divided into three groups according to whether this nutrilite was essential, stimulating or relatively unimportant. Beta-alanine was able to replace pantothenic acid in providing the combined growth factor effect.

In the case of three species inositol was found necessary for optimum growth, this nutrilite being specially important for *Zygosaccharomyces nectarophilus*, though effective only in the presence of biotin and pantothenic acid. With yeasts not requiring inositol the combined growth factor effect could be obtained with biotin and pantothenic acid.

In some cases thiamin exerted slightly stimulating or depressing effects, being rather more significant in promoting maximum growth in the case of *Zygosaccharomyces nussbaumeri*, *Z. richteri* and one strain of *Z. nectarophilus*. Pyridoxin was still less important as a nutrilite for osmophilic yeasts.

Strains previously considered specifically identical on the basis of morphology and fermentative capacity showed good agreement in nutrilite requirements, suggesting the value of the latter determination as an aid in classification.

REFERENCES

Church, M. B., Paine, H. S., and Hamilton, J. 1927 Sugar-tolerant yeasts in chocolate-coated creams. Ind. Eng. Chem., **19**, 353–357.

Eakin, R. E., and Williams, R. J. 1939 Vitamin B_6 as a yeast nutrilite. J. Am. Chem. Soc., **61**, 1932.

Eastcott, E. V. 1928 Wildier's Bios. The isolation and identification of "bios I". J. Phys. Chem., **32**, 1094–1111.

Eastcott, E. V. 1941 Toxicity and reproduction of yeast. Can. Chem. and Process Ind., **25**, 308–312.

Fabian, F. W., and Hall, H. H. 1933 Yeasts found in fermented maple syrup. Zentr. Bakt. Parasitenk., II, **89**, 31–47.

Fabian, F. W., and Quinet, R. I. 1928 A study of the cause of honey fermentation. Tech. Bull. No. 92, Mich. State Coll. Agr. Expt. Sta.

Farrell, Leone 1935 The influence of inositol, Bios IIA, and Bios IIB on the reproduc-

tion of twelve species of yeast. A new constituent of Bios. Trans. Roy. Soc. Can. III, **29**, 167–173.

Farrell, Leone, and Lochhead, A. G. 1931 Accessory food substances for osmophilic yeasts. II. Comparison of honey bioactivator with Bios. Can. J. Research, **5**, 539–543.

Hall, H. H., James, L. H., and Nelson, E. K. 1937 Microorganisms causing fermentation flavors in cane syrups, especially Barbados "molasses." J. Bact., **33**, 577–585.

Kögl, F., and Tonnis, B. 1936 Über das Bios-Problem. Darstellung von krystallisiertem Biotin aus Eigelb. Z. physiol. Chem., **242**, 43–73.

Kroemer, K., and Krumbholz, G. 1931 Untersuchungen über osmophile Sprosspilze. Arch. Mikrobiol., **2**, 352–410.

Lochhead, A. G., and Farrell, Leone 1931 The types of osmophilic yeasts found in normal honey and their relation to fermentation. Can. J. Research, **5**, 665–672.

Lochhead, A. G., and Heron, D. A. 1929 Microbiological studies of honey. I. Honey fermentation and its cause. II. Infection of honey by sugar-tolerant yeasts. Can. Dept. Agr. Bull. No. 116.

Marvin, G. E. 1928 The occurrence and characteristics of certain yeasts found in fermented honey. J. Econ. Entomol., **31**, 363–370.

Mrak, E. M., and Baker, E. E. 1940 Sugar-tolerant yeasts causing the spoilage of dried fruits. Proc. 3rd Intern. Congr. Microbiol. 1939, 707–708.

Nussbaumer, Th. 1910 Beitrag zur Kenntnis der Honiggärung nebst Notizen über die chemische Zusammensetzung des Honigs. Z. Untersuch. Nahr. Genuss., **30**, 272–277.

Owen, W. L. 1913 The occurrence of *Saccharomyces zopfii* in cane syrup and variation in its resistance to high temperatures when grown in solutions of varying densities. Zentr. Bakt. Parasitenk., II, **39**, 468–482.

Rainbow, C. 1939 The bios requirements of various strains of *Saccharomyces cerevisiae.* J. Inst. Brewing, **45**, 533–545.

Richter, A. A. 1912 Über einen osmophilen Organismus, den Hefepilz *Zygosaccharomyces mellis acidi* sp. n. Mycolog. Centr., **1**, 67–76.

Sacchetti, M. 1932a I lieviti della fermentazione del miele italiano. Mem. accad. sci. ist. Bologna, Classe sci. fis. Ser. VIII, **9**, 1–11.

Sacchetti, M. 1932b Ricerce sulla fermentazione di un mosto d'uva concentrato. Arch. Mikrobiol., **3**, 473–491.

Schultz, A. S., Atkin, L., and Frey, C. N. 1939 Vitamin B_6, a growth-promoting factor for yeast. J. Am. Chem. Soc., **61**, 1931.

Schultz, A. S., Atkin, L., and Frey, C. N. 1940 The biochemical classification of yeast strains. J. Bact., **40**, 339–346.

Shutt, D. B. 1925 Yeast contamination as a source of explosion in chocolate coated candies. Sci. Agr., **6**, 118–119.

Wildiers, E. 1901 Nouvelle substance indispensable au developpement de la levure. La Cellule, **18**, 313–331.

Williams, R. J. 1919 The vitamine requirement of yeast, a simple biological test for vitamine. J. Biol. Chem., **38**, 465–486.

Williams, R. J. 1941 Growth-promoting nutrilites for yeasts. Biol. Rev. Cambridge Phil. Soc., **16**, 49–80.

Williams, R. J., Eakin, R. E., and Snell, E. E. 1940 The relationship of inositol, thiamin, biotin, pantothenic acid and vitamin B_6 on the growth of yeasts. J. Am. Chem. Soc., **62**, 1204–1207.

Williams, R. J., Lyman, C. M., Goodyear, G. E., Truesdail, J. H., and Holaday, D. 1933 "Pantothenic acid", a growth determinant of universal biological occurrence. J. Am. Chem. Soc., **55**, 2912–2927.

Williams, R. J., and Roehm, R. R. 1930 The effect of antineuritic vitamin preparations on the growth of yeasts. J. Biol. Chem., **87**, 581–590.

Williams, R. J., and Rohrmann, E. 1936 β-Alanine and "bios." J. Am. Chem. Soc., **58**, 695.

VALIDITY OF THE GENUS ALCALIGENES

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A recent paper by Conn, Wolfe and Ford (1939) discussed the question as to whether certain soil bacteria are related to the genus *Alcaligenes* as recognized in recent editions of Bergey's Manual of Determinative Bacteriology. So far as concerns the relationships between the soil bacteria and plant parasites discussed in that paper, the reasoning still seems sound. In one or two particulars, however, modification of the conclusions seems necessary in light of further evidence that has been obtained. This evidence relates to the identity of the type species, *Alcaligenes faecalis*. The statement was made in that paper: "Apparently, therefore, there is a commonly recognized organism to which the name *Alcaligenes faecalis* can be assigned." Evidence is now at hand which makes that statement doubtful. Before presenting this evidence, however, a few words are necessary as to the history of this name, and of the specific designation.

HISTORICAL

Petruschky (1896) applied the name *Bacillus faecalis alcaligenes* to a peritrichous, non-spore-forming rod of intestinal origin, producing no acid in sugar broths and causing alkalinity in milk; no detailed description was given. It was renamed *Bacillus alcaligenes* by Migula (1900) and *Alcaligenes faecalis* by Castellanni and Chalmers (1919). This generic name has been criticized as being an adjective instead of a noun, thus having been formed contrary to a recommendation of authorities on nomenclature. It can, however, hardly be called invalid on this account and was accepted in the first edition of Bergey's Manual, with the following definition:

"Motile or non-motile rods, generally occurring in the intestinal canal of normal animals. Do not form acetyl-methyl-carbinol. Do not ferment any of the carbohydrates."

In this edition of Bergey's Manual eight other species were included with it, only two of which were stated to occur in the intestinal canal; three were described as milk or udder bacteria; three (*A. melitensis*, *A. abortus*, and *A. bronchisepticus*) have since been transferred to the genus *Brucella*, one becoming the type species of the latter.

The genus *Brucella* was first recognized in the fourth edition of the Manual, and the first two of the three above mentioned species were then transferred to this other genus. The number of *Alcaligenes* species recognized, however, was

[1] Acknowledgement is made to Jean E. Conn and to Mark Ford for much of the data upon which this paper is based.

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now ten, since additional species had been placed in it; just half of these ten species are intestinal forms.

In the fifth edition of the Manual (1939) *A. bronchisepticus* was transferred to the genus *Brucella* because of protests of students of the latter group, and one soil form (*A. radiobacter*) was added; the latter had not been included in any genus in earlier editions of the Manual and it was now placed in *Alcaligenes* because this was the only genus whose description it would fit. In this last edition of the Manual, the writer undertook to revise the definition of the genus and the statement concerning habitat was made to read "which generally occur in the intestinal canal, in decaying materials, dairy products and soil." Just half of the species were still described as intestinal forms.

The writer first became interested in this genus when it proved that it was the only place in the Bergey system (until, in the 5th edition, *Bacterium* was again recognized) where the numerous soil bacteria which do not ferment sugars and tend to produce alkalinity in milk can be placed. Recognition of this fact raised two questions which have not proved easy to answer: (1) Are the soil forms and intestinal forms, which do not produce acid or gas from sugars, closely related to each other? (2) Can the type species *Alcaligenes faecalis* be recognized?

THEORETICAL

There is one theoretical point which must be first discussed, because failure to recognize it can cause considerable confusion in the understanding of organisms similar to these under discussion.

When an organism produces acid and gas from a sugar, or even when it produces no gas but sufficient acid to be detectable in the presence of peptone, there is no question as to its fermentative ability. When, however, neither acid nor gas is evident on a peptone-sugar medium, there is danger of confusion. Often all such organisms are grouped together as "inert" or "non-fermenting" bacteria. Actually, however, there may be two distinctly different classes included in this category; (1) organisms which are unable to use sugar; (2) organisms which utilize sugar (especially glucose) so completely that there are no by-products, and the only end-product capable of giving an acid reaction to the medium is carbon dioxide—a change in reaction which can be detected only on media of low buffer content. It will be seen that theoretically these two classes of bacteria stand far apart in physiology and yet the generally recognized tests for fermentation would group them together.

SIMILARITY TO SOIL FORMS

As explained above, one of the questions to be answered is: Are the soil forms and the intestinal forms which produce no acid from sugars closely related? To answer this question, a series of soil and intestinal forms were collected.

For the soil forms, *Bacterium radiobacter* Löhnis was selected as typical. Included with it were miscellaneous short rod forms isolated from soil in the writer's laboratory.

To secure intestinal forms known to be *Alcaligenes faecalis* proved difficult.

Four cultures under this name were secured from the American Type Culture Collection; one of them was quickly discarded, as it proved to have been overgrown by one of the fluorescent pseudomonads. Four were obtained from the National Collection of Type Cultures at the Lister Institute. A collection of 14 cultures was obtained from the laboratory of Dr. Carl Nyberg in Helsinki, 5 of which were labelled "*A. faecalis*" the others "similar to *A. faecalis.*" Two strains were obtained from Dr. Pribram at Chicago. A series of 11 cultures was secured from Dr. E. G. D. Murray of Montreal; these were freshly isolated non-acid-formers, but were not all regarded as typical representatives of this species. A strain was obtained from the University of Rochester Medical School, which was a stock culture that had been used for illustrative purposes in class work for some time, but whose origin was not known. Of these various cultures, a few proved to be lophotrichous; they obviously must belong elsewhere, as this species was originally described as peritrichous and has generally been accepted as such.

The difficulty in securing authentic cultures of this species seems to arise from the fact that bacteriologists are not specially interested in it. Any non-fermenting culture of fecal origin is regarded as *Alcaligenes faecalis* and of no significance; hence the culture is discarded without thought of preserving it as a stock culture for comparison with authentic strains.

In comparing the soil forms with the intestinal forms only a few characteristics proved available for making distinctions. The morphology did not vary; acid and gas tests on ordinary media are always negative; production of nitrite from nitrate proved a variable character. Two features, however, did prove useful: (1) ability to grow on synthetic media; (2) production of CO_2 from glucose. The latter character seemed particularly worth investigating, as it has been shown by Conn and Darrow (1935) and by Hofer (1941) that such soil forms as *Bacterium globiforme* and *Bacterium radiobacter*, although producing no organic acid from glucose, do give rise to very appreciable quantities of carbon dioxide.

Ability to grow on synthetic media. To test this point two synthetic media were prepared, of the following composition:

Medium I

$(NH_4)_2HPO_4$	1.0 g.
KCl	0.2
$MgSO_4 \cdot 7H_2O$	0.2
Yeast extract	0.2
Agar	15
H_2O	1000 ml.

Medium II

Same but with the addition of glucose	1.5 g.

The soil forms showed scanty growth on Medium I, but very vigorous growth on Medium II. Only five of the cultures received as examples of *Alcaligenes faecalis* were the same in this respect. Two of these five were from the collection received from Montreal, but showed evidence of contamination. The other three were the strains obtained from the American Type Culture Collection and on

the basis of which the earlier conclusions were drawn; two of them were reported to have come originally from Dr. E. O. Jordan, the other (isolated from blood) from Dr. Alice C. Evans.

Three of the strains obtained from Dr. Nyberg showed fair growth in both media, no better with glucose than without; but these three strains were among those labelled by him as merely "*similar* to *A. faecalis*."

TABLE 1

CO_2- Production in 20 ml. of ammonium phosphate media

ORGANISM	SOURCE	CO_2 FROM 30 MGM. GLUCOSE		
		Medium III, complete	Medium IV, no yeast extract	Medium V, no glucose
		mgm.	*mgm.*	*mgm.*
B. radiobacter	Hofer	27.5	23.2	
?	Soil		14.0	
?	Soil		12.5	
A. faecalis 1	ATCC	17.38	15.84	0.88
A. faecalis 2	ATCC	16.72	15.73	
A. faecalis 3	ATCC	4.62	2.75	0.88
A. faecalis 4	U. of Rochester	1.43	0.11	0.22
A. faecalis 5	Murray	1.76	0.55	
A. faecalis 6	Murray	4.62	1.21	
A. faecalis 7*	Murray	20.24	22.84	
A. faecalis 8	Murray	5.39	? †	
A. faecalis 9	Murray	0.66	1.65	
A. faecalis 10	Murray	0.66	1.43	
A. faecalis 11	Murray	5.06	2.42	0.44
A. faecalis 12	Murray	0.55	0.33	
A. faecalis 13*	Murray	19.69	4.62	
A. faecalis 14	Murray	4.62	0.44	0.22
A. faecalis 16	Lister Inst.	2.86	2.42	
A. faecalis 17	Lister Inst.	1.54	1.98	
A. faecalis 18	Lister Inst.	2.75	0.22	
A. faecalis 19	Lister Inst.	2.20	1.54	
A. faecalis 20	Pribram	2.75	0.99	0.44
A. faecalis 22	Nyberg‡	2.42	0.44	
A. faecalis 23	Nyberg	21.45	9.46	0.88

* Culture apparently overgrown by contaminant before this test was made.
† Disagreement of duplicates.
‡ See Nyberg (1935).

All the rest of the strains showed poor or no growth in either medium. Among them were included: Nine of the cultures from Dr. Murray; 4 from the Lister Institute; the University of Rochester culture, and the 2 from Dr. Pribram; all 4 of the cultures from Dr. Nyberg claimed to be authentic, and 6 of those submitted by him as being similar organisms.

The weight of evidence from this comparison of cultures is that *A. faecalis*, as generally recognized, does not grow on the particular synthetic medium selected,

and unlike the soil forms, cannot make use of glucose on such a medium. There is no evidence at hand as yet to indicate that *A. faecalis*, as represented by the bulk of cultures received, can grow without organic nitrogen. Apparently strains from the American Type Culture Collection are a different organism.

Production of CO_2 from glucose. This problem was investigated by the Eldredge tube technic. The media employed were as follows:

Medium III: Like medium I above but without agar.

Medium IV: Like Medium III but without yeast extract.

Medium V: Like III but without glucose (used merely as a check on 7 of the cultures). The results, with 21 strains of the so-called "*A. faecalis*", in comparison with similar determinations for certain soil forms are given in table 1. It will be seen that abundant CO_2 is produced by the soil forms. The only "*A. faecalis*" cultures, however, which showed appreciable CO_2 in the absence of yeast-extract were two of the three from the American Type Culture Collection and one or two others of doubtful purity.[3]

CONCLUSIONS

It is evident that the similarity of *A. faecalis* to certain soil bacteria which was previously pointed out by Conn, Wolfe, and Ford, may not actually exist; in pointing out this similarity, too much significance was attached to the cultures received from the American Type Culture Collection. If instead of regarding these cultures as representative of the species, one accepts as typical those obtained from other collections, very different conclusions must be drawn. The cultures from the former source *are* very much like the soil forms; but a majority of those from other sources require organic nitrogen and show no evidence of utilizing glucose—while the soil forms in question utilize inorganic nitrogen and glucose, using the latter so economically and completely that they do not produce appreciable acid in ordinary media. Following the scheme of classification of the Eubacteriales given on pp. 20–21 of the 5th edition of Bergey's Manual, this would put the soil forms in a widely different section of this Order from that where one would have to place most of the *A. faecalis* cultures studied (except those from the American Type Culture Collection).

These findings also raise serious question as to whether anyone knows just what *Alcaligenes faecalis* is. The original description by Petruschky is vague; and even the recent emendations are incomplete, as evidenced by the fact that the published descriptions would fit either the type growing on synthetic media or that which requires organic nitrogen. Both of these types are being distributed under the same name; and it is difficult, if not impossible, to decide which is authentic. The writer of this paper, as a soil bacteriologist, hardly feels competent to venture an opinion.

[3] Abundant CO_2 production from all of these cultures has been observed in a veal-infusion medium containing glucose. This, however, is of no significance, as the break-down of veal infusion has been shown to yield CO_2, and those of the organisms included in this work which were tested on this same medium without glucose were found to give off almost, if not quite, as much of this gas as in its presence.

Still further evidence that bacteriologists are in disagreement as to the characteristics of this species is furnished by the fact that at least one of the cultures furnished under this name proves to have polar flagella. This organism can hardly be the true *A. faecalis*, as this species has always been described (even by Petruschky) as being peritrichous; but the fact that it was obtained under that name is further indication of the confusion that exists.

TAXONOMIC RECOMMENDATIONS

The above data seem to invalidate the recent recommendations, made in part by the author and associates, that some of these soil bacteria belong in the genus *Alcaligenes*. The same objection can be raised to including the other non-parasitic forms which have been put in the genus—notably the species *Alcaligenes viscosus*, which proves to be closely related to the soil types under consideration. If the three species occurring in milk and soil, listed in this genus in the last edition of Bergey's Manual, be removed from it, there remain six parasitic species, including the type species *A. faecalis*. Three pertinent questions arise: (1) Should the Genus *Alcaligenes* be retained for these six species? (2) If not, in what genus should they be placed? (3) In any event, what should be done with the soil and milk forms now bearing this generic name?

The writer wishes to venture the following opinions:

1. The genus *Alcaligenes* can be retained only if its type species can be recognized. As explained above, at least two different organisms are being distributed by culture collections under the name *A. faecalis*. Although it seems entirely probable that the true intestinal type is the one which requires organic nitrogen, the matter certainly needs clarification. It is apparently impossible to determine the exact nature of the original organism studied by Petruschky; and general agreement as to the nature of the species would be very difficult to secure under present world conditions. Hence, the final decision may be to drop the name entirely. Further justification for doing this could be found in the fact that the generic name is an adjective, not a noun as recommended in codes of nomenclature.

2. There are at present five intestinal inhabitants listed under this genus in Bergey's Manual: *A. faecalis*, *A. metalcaligenes*, *A. ammoniagenes*, *A. bookeri* and *A. recti*. These might well make a genus by themselves, especially if it proves that *A. faecalis* is the organism which requires organic nitrogen, and that the other species are like it in this respect.

Undoubtedly, the most convenient arrangement, in the next revision of Bergey's Manual, will be to leave them in this genus, with the thought of its being a temporary grouping until further study of species included can be made. It is clear that all five species, even the type species, require such study before their identity can be definitely learned. Meanwhile those who are unwilling to put them in a genus whose type species is not definitely recognized can call them *Bacterium alcaligenes*, *B. metalcaligenes*, *B. ammoniagenes*, *B. bookeri*, and *B. recti*, respectively. None of these names are new combinations, and the definition of *Bacterium* in the 5th edition of the Manual is sufficiently broad to cover these organisms.

3. At present only one soil organism has been placed in *Alcaligenes*, namely *A. radiobacter*. This organism is plainly out of place in the genus if the type species is the intestinal organism which requires organic nitrogen. It is, on the other hand, very similar to the legume nodule organisms, *Rhizobium spp.* and to the bacteria causing crown gall and hairy root (now in *Phytomonas*). Students of these groups are coming to think that all these species should be close together in the classification. The legume nodule organisms undoubtedly constitute a definite and easily recognized genus; but after consultation with bacteriologists who have studied both the genus *Rhizobium*, and the closely related plant pathogens, it seems that a new genus is called for. This genus should contain the plant pathogens closely related to the crown gall organism, the soil species now in *Alcaligenes*, and other saprophytic organisms that may be found to be similar in morphology and physiology. For this new genus the name *Agrobacterium* is now proposed, with *A. tumefaciens* as its type species[4]. For this genus the following description is hereby given:

Agrobacterium. Small, short, non-spore-forming rods, which are typically motile with 1–4 peritrichous flagella; (if only one flagellum, lateral attachment is as common as polar). Occur primarily in the soil or as pathogens attacking roots or producing hypertrophies on the stems of plants. Are ordinarily gram-negative. Do not produce acid or gas[5] in glucose-peptone media, although a certain amount of acid is often evident in synthetic media; this latter observation is ordinarily due merely to presence of CO_2 which may be produced in considerable abundance. Liquefy gelatin slowly or not at all.

The type species *Agrobacterium tumefaciens* is the cause of crown-gall of plants, and may be described as in the 5th edition of Bergey's Manual. Placed with this should be *Agrobacterium radiobacter* (syn. *Bacterium radiobacter*, *Achromobacter radiobacter*, *Alcaligenes radiobacter*)[6] and *Agrobacterium rhizogenes* (syn. *Phytomonas rhizogenes*), the cause of hairy root, both described as in the 5th edition of Bergey.

[4] In Bergey's Manual *Polymonas tumefaciens* (Lieske, 1928) is given among the synonyms of this species, and would seem to be called for by rules of priority. This is not the case, however, because a consultation of Lieske's paper shows that it is not a true synonym. Lieske was of the school who believed crown-gall to be identical with human and animal cancer, and to be caused by a microorganism. The above-cited name he assigns to a polymorphic organism which he claims to be the cause of cancer, and he adds that *Bacterium tumefaciens* is one phase of this organism. His theory is not accepted today, and no organism is known which possesses the pleomorphic characteristics he assigns to *Polymonas tumefaciens*.

[5] In this definition the expression "acid or gas" is used in the rather indefinite sense in which it is ordinarily employed in manuals of determinative bacteriology; thus "acid" essentially means a sufficient change in reaction to be detected by litmus, and "gas" means sufficient gas to be visible as a bubble in the liquid. Hence the statement "do not produce acid or gas" does not exclude the organisms which produce CO_2 without saturating the medium with the gas or lowering its pH beneath approximately 6.4, nor those which produce organic acids without greater change in pH than this.

[6] Hofer (1941) mentions as a possible synonym of this organism *Pseudohizobium ramosum* Hartleb, 1900. This name would have priority if it could stand; but Hartleb's description is too vague for recognition, and it seems very unwise to emend the description so that it could be recognized—for this would mean abandoning the specific as well as the generic name under which this organism has long been known.

Of the milk forms in the genus *Alcaligenes* the only one which seems definitely recognizable is *Alcaligenes viscosum*. This seems to lie closer to those species discussed in the preceding paragraph than it does to the intestinal forms which require organic nitrogen; until its relationships are more definitely established it can be called *Bacterium viscosum* after Weldin and Levine (1923). The other milk species are very poorly known and have never been put in the genus *Bacterium* except under the trinomials *Bacterium lactis album* and *Bacterium lactis marshalli*. It is recommended that these be transferred together with the equally poorly known species *Alcaligenes dernieri* (obtained from rubber latex) to an appendix of indefinitely described species.

For the present the writer prefers to make no further new combinations by putting other species in this new genus. There are several other plant pathogens, listed in Appendix II of *Phytomonas* in the 5th edition of Bergey's Manual, which probably belong in the same genus with the crown-gall organism; but it seems well to leave it for phytopathologists to rename them if they are to be transferred to *Agrobacterium*. There are, moreover, various soil species, some of which have been named and others still unnamed, which seem equally closely related to the species which it is now proposed to call *Agrobacterium radiobacter*. It is hoped that the new genus will prove a convenient place for some forms which do not fit well into any of the genera recognized in past editions of Bergey's Manual; it is also hoped that those adding species to this genus will include only those types covered by the above definition.

REFERENCES

Bergey, D. H. et al. 1939 Manual of Determinative Bacteriology. 5th ed. Baltimore, Williams & Wilkins Co.

Castellani, A., and Chalmers, A. 1919 Manual of Tropical Medicine. 3rd ed. London, Ballière, Tyndall and Cox.

Conn, H. J., and Darrow, Mary A. 1935. Characteristics of certain bacteria belonging to the autochthonous microflora of soil. Soil Sci., **39**, 95–110.

Conn, H. J., Wolfe, Gladys E., and Ford, M. 1940 Taxonomic relationships of *Alcaligenes spp.* to certain soil saprophytes and plant parasites. J. Bact., **39**, 207–226.

Hartleb, R. 1900 Die Morphologie und systematische Stellung der sogenannten Knöllchenbakterien. Chem. Ztg., **24**, 2d. sem. 887–888.

Hofer, A. W. 1941 A characterization of *Bacterium radiobacter* (Beijerinck and Van Delden) Löhnis. J. Bact., **41**, 193–224.

Lieske, R. 1928 Untersuchungen über die Krebskrankheit bei Pflanzen, Tieren, und Menschen. Zentr. Bakt. Parasitenk., I, Orig., **19**, 118–146.

Migula, W. 1909 System der Bakterien. Fischer, Jena., **2**, 737.

Nyberg, C. 1935 Bacillus faecalis alcaligenes Petruschky. Zentr. Bakt. Parasitenk., I, Orig., **133**, 443–449.

Petruschky, J. 1896 *Bacillus faecalis alcaligenes* (n.sp.). Zentr. Bakt. Parasitenk., I, **19**, 187–91.

BACTERIAL MORPHOLOGY AS SHOWN BY THE ELECTRON MICROSCOPE

III. Cell-Wall and Protoplasm in a Strain of Fusobacterium

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The differentiation of a solid bacterial cell-wall from an inner protoplasm has been demonstrated in favorable cases by plasmolysis, by microdissection, and by differential staining; notable success with mordant and differential staining has recently been attained by Knaysi (1938, 1941); the literature is reviewed by Lewis (1941). Examination of a variety of bacterial species with the aid of the electron microscope has demonstrated this important structural differentiation with particular vividness. The general conclusion is warranted, we believe, that bacteria are cells, with solid cell-wall clearly distinct from inner fluid or potentially fluid protoplasm. The inner protoplasm is frequently observed to be shrunken from the cell-wall by plasmolysis or drying, and readily escapes from the cell-wall following injury. The capacity of undergoing reversible gelation is possessed by protoplasm in general. We know of no convincing evidence, however, as to whether normal bacterial protoplasm is a sol or a gel or whether it may undergo reversible change from one state to the other.

Bacteria of the genera thus far considered in this series, *Streptococcus*, (Mudd and Lackman, 1941), and *Bacillus*, (Mudd, Polevitzky, Anderson, and Chambers, 1941), have been relatively opaque to the electron beam. In the present study, pictures of a strain of fusiform bacillus are presented in which both differentiation of cell-wall from protoplasm and differences of density within the protoplasm itself are particularly clearly shown.

The strain of fusiform bacillus, isolated by Wakeford, the morphology of whose cells is shown herewith, has been described, (Kast, 1928). This strain has been preserved in coagulated serum medium for some thirteen years, and has maintained its characteristic morphology during this time. For the present study transplants were made to cystein-serum broth medium, sealed with vaseline, and incubated for various periods of time. For examination with the electron microscope a small amount of culture was removed with a capillary pipette and suspended in distilled water, centrifugalized, and the sediment resuspended in distilled water; this washing procedure was twice repeated. A droplet of the last suspension in distilled water was placed on the collodion mount and allowed to dry without fixing or staining. All pictures were taken with electrons accelerated by 60 Kilovolts and at an original magnification of 6250 diameters.

[1] RCA Fellow on the Electron Microscope, National Research Council.

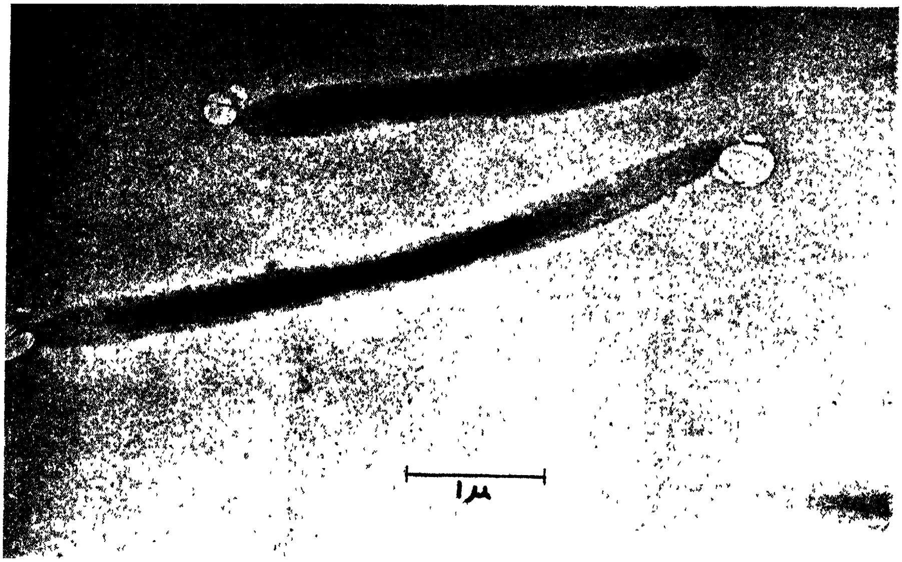

FIG. 1. TWO CELLS FROM YOUNG (2 DAYS OLD) CULTURE
Final magnification as reproduced × 17,500

FIG. 2. BRANCHING FORM FROM YOUNG (2 DAYS OLD) CULTURE
Washed bacteria had stood in distilled water overnight. Final magnification × 19,000

In figure 1, two characteristic cells of a young culture are seen. Differences in density are obvious in various parts of the protoplasm. A branching form

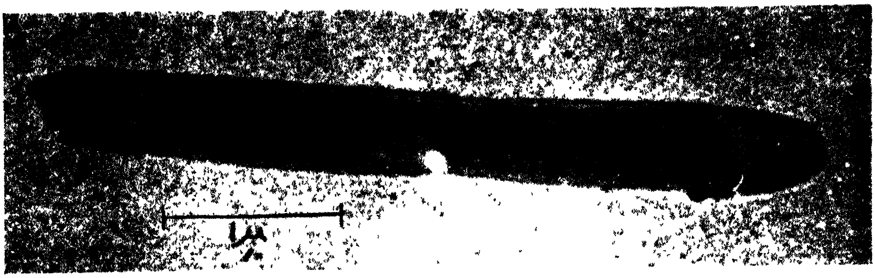

FIG. 3 CELL FROM CULTURE INCUBATED 48 HOURS AND KEPT AT ROOM TEMPERATURE FOR 2 WEEKS × 23,500

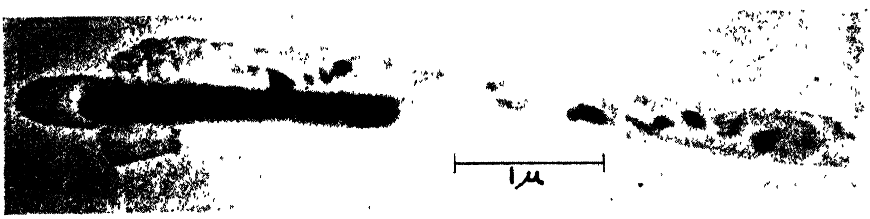

FIG. 4 CELLS FROM CULTURE TREATED AS IN FIGURE 3 × 19,500

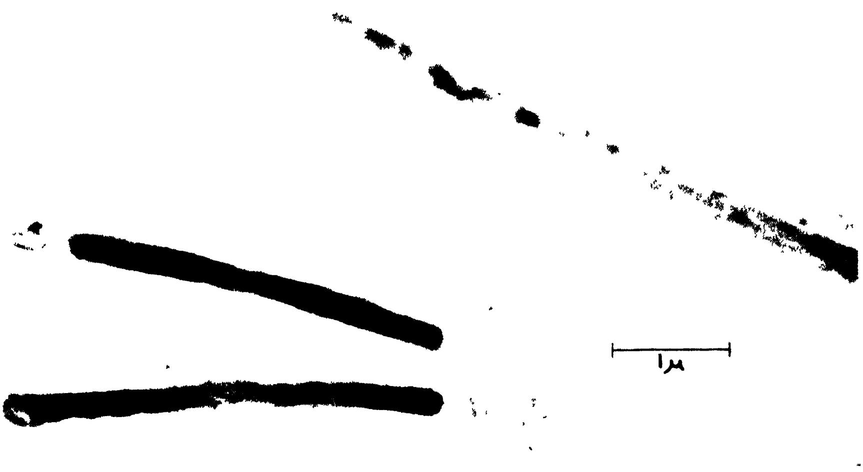

FIG. 5. CELLS FROM CULTURE TREATED AS IN FIGURE 3 × 15,500

is shown in figure 2. A cell from an older culture appears in figure 3. In this cell very dense local areas appear as black granules against a background of relatively "transparent" protoplasm which is retracted from the cell-wall.

Figure 4 is of a preparation from a two-weeks old culture. The end of the cell-wall of one cell has been broken: the jagged line of rupture attests to the solidity of the cell-wall, (as in the previous studies in which fracture by sonic vibration

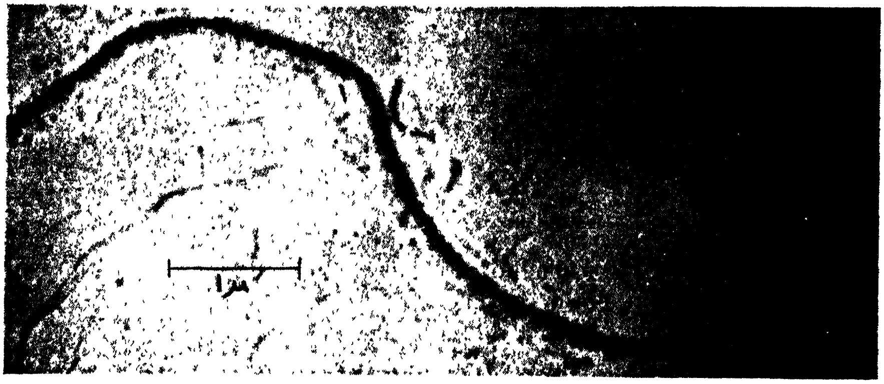

FIG. 6. CURVED FILAMENTOUS BACTERIUM FROM A CULTURE TREATED AS IN FIGURE 3 × 17,000

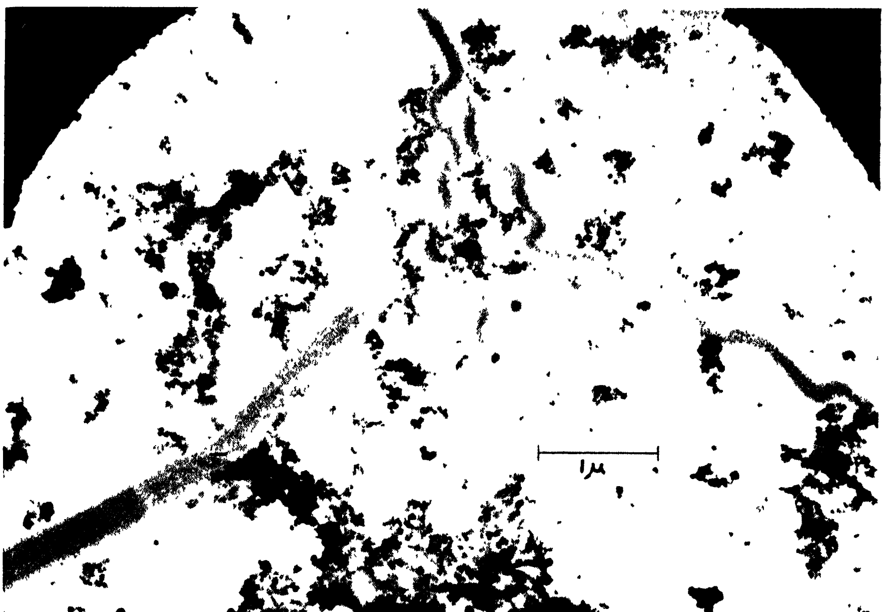

FIG. 7. EXUDATE FROM INFECTED AREA OF A CASE OF VINCENT'S GINGIVITIS OF TWO WEEKS DURATION × 15,500

was employed). The other cell appears to be intact. Its relatively dense protoplasm is retracted from the cell-wall. In figure 5 cells from a two-weeks old culture are again shown. In two of the cells the protoplasm is relatively dense. The tapering ends of the cells are apparently free of protoplasm and the cell-

wall appears to be wrinkled in several places. Part of a larger filamentous cell is also shown, in which dark granules appear in a protoplasm which is relatively transparent to the electron beam. Figure 6 shows a curved, filamentous form found in an old culture of the fusiform bacillus. It is a filamentous bacterial form which might easily be mistaken for a spirochete if studied with the light microscope.

As a matter of interest, a fresh preparation of the exudate from a case of Vincent's gingivitis is shown in figure 7. Fusiform and spiral microorganisms are shown in association.

DISCUSSION

The differentiation of cell-wall from inner protoplasm has been clearly demonstrated in electron micrographs of streptococci (Mudd and Lackman, 1941), of species of the genus *Bacillus* (Mudd, Polevitzky, Anderson, and Chambers, 1941), and in *Thiobacillus thiooxidans* (Umbreit and Anderson, 1942). Cell-wall and inner protoplasm appear as structurally distinct, also, in many of the electron micrographs in the German literature (Piekarski and Ruska, 1939; Jakob and Mahl, 1940), but have not always been correctly interpreted. The differentiation of cell-wall from protoplasm has also been demonstrated by combination of the protoplasm with salts of silver and lead, resulting in greatly increased density of the protoplasm without discernible alteration of the cell-wall (Mudd and Anderson, 1942).

The differences in density of the protoplasm of these unfixed (though dried) and unstained cells of *Fusobacterium* were not unexpected, in view of the uneven staining which has been described as characteristic of fusiform bacilli (Varney, 1927; Hine and Berry, 1937; Bergey's Manual, 1939). Moreover, when the living cells are observed with dark field apparatus[2] they show granular structures essentially similar to those more clearly shown in the electron micrographs. Exact correlation between density as found with the electron microscope and staining behavior has not been attempted in this study, (see, however, Piekarski and Ruska, 1939).

CONCLUSION

Bacteria are cells with solid cell-wall and fluid, or potentially fluid, inner protoplasm distinct from the cell-wall. Electron micrographs of cells of a strain of *Fusobacterium* show striking differences in density within the protoplasm. Correlation of the significance of the differentiations observable with the electron microscope with those observable by microchemical and staining techniques, though hardly more than begun, presents a challenging problem.

REFERENCES

Bergey, D. H. 1939 Manual of Determinative Bacteriology. 5th Ed. Baltimore, p. 586.
Hine, M. K., and Berry, G. P. 1937 Morphological and cultural studies of the genus *Fusiformis*. J. Bact., **34**, 517–533.

[2] Zeiss "Bitumi" binocular attachment, cardioid condenser, quartz objective, carbon arc, magnification approximately × 1000.

Jakob, A., and Mahl, H. 1940 Strukturdarstellung bei Bakterien, insbesondere die Kapseldarstellung bei Anaerobiern mit dem elektrostatischen Elektronen-Übermikroskop. Arch. exptl. Zellforsch. Gewebezücht., **29**, 97–104.

Kast, C. C. 1928 Observations on the morphology and motility of fusiform bacilli. J. Lab. Clin. Med., **14**, 112–119.

Knaysi, G. 1938 Cytology of bacteria. Botan. Rev., **4**, 83–112.

Knaysi, G. 1941 Observations on the cell division of some yeasts and bacteria. J. Bact., **41**, 141–154.

Lewis, I. M. 1941 The cytology of bacteria. Bact. Revs. **5**, 181–230.

Mudd, S., and Anderson, T. F. 1942 Differential "staining" for electron microscopy. J. Exptl. Med., in press.

Mudd, S., and Lackman, D. B. 1941 Bacterial morphology as shown by the electron microscope. I. Structural differentiation within the streptococcal cell. J. Bact., **41**, 415–420.

Mudd, S., Polevitzky, K., Anderson, T. F., and Chambers, L. A. 1941 Bacterial morphology as shown by the electron microscope. II. The bacterial cell-wall in the genus *Bacillus*. J. Bact., **42**, 251–264.

Piekarski, G., and Ruska, H. 1939 Übermikroskopische Untersuchungen an Bakterien unter besonderer Berücksichtigung der sogenannten Nucleoide. Arch. Mikrobiol., **10**, 302–321.

Umbreit, W. W., and Anderson, T. F. 1942 A study of *Thiobacillus thiooxidans* with the electron microscope. J. Bact., **44**, 317–320.

Varney, P. L. 1927 The serological classification of fusiform bacilli. J. Bact., **13**, 275–314.

RELATIVE INHIBITION OF MICROÖRGANISMS BY GLUCOSE AND SUCROSE SIRUPS[1]

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In concentrations above 30 per cent, sugar has a significantly inhibitive effect on microörganisms, which causes it to be considered as a preservative as well as a sweetening agent.

Sucrose and glucose have molecular weights of 342 and 180, respectively. Therefore a given quantity of glucose in solution should result in a greater osmotic pressure than a sucrose solution of the same concentration. Theoretically, the osmotic pressure of glucose is 1.73 times that of sucrose. Hunziker (1935) concluded that glucose in concentrations used in the manufacture of condensed milk was about 1.25 times as effective as sucrose in the preservation of this product. However, Berkeley and Hortley (1906) found that as the concentrations of the solution are increased, the osmotic pressure of a sucrose solution will approach that of a glucose solution. Ramsey, Tracy, and Ruehe (1933) suggest this to be due to the greater affinity of the sucrose particle for the water molecule with a consequent increase in concentration and osmotic pressure.

Urbain and Miller (1930) found that glucose-treated yolk batters resisted fermentations and bacterial decomposition to a greater extent than those batters to which sucrose had been added. This difference was attributed to the greater osmotic pressure developed by the monosaccharide. Buchanan (1932) reported that, on heating, yeast spores were killed more readily in a sucrose solution than in a glucose solution of the same concentration. Fellers, Miller, and Onsdorff (1937) state that glucose sirups are considered to be more effective than sucrose sirups in the inhibition of microörganisms. They found no more spoilage in fruit products containing glucose than in those containing only sucrose.

EXPERIMENTAL

The plan of this investigation was formulated to study the effect of glucose, sucrose, and mixtures of both sugars on yeasts and molds which are microörganisms often associated with the spoilage of foods of high sugar concentrations. The index organisms selected were the common bakers' yeast, *Saccharomyces cerevisiae* and the mold, *Aspergillus niger*. Although the different species of yeasts and molds may vary extensively one from the other, in many of their activities, the results obtained with *Saccharomyces cerevisiae* and *Aspergillus niger* should indicate the general trend of inhibition of other closely related microörganisms by these sugars.

[1] Contribution No. 423, Massachusetts Agricultural Experiment Station.

Methods

The sugars used in these experiments were glucose and sucrose. The glucose was commercial anhydrous dextrose and the sucrose was commercial refined cane sugar. To prevent hydrolysis all sugar solutions were sterilized without heat by passage through a Seitz filter. Erlenmeyer flasks of 250 ml. capacity were used each containing 50 ml. of the sugar solution tested. Mineral nutrients (0.8% $(NH_4)_2SO_4$, 0.1% $CaCl_2$, 0.15% KH_2PO_4, 0.2% KNO_3, 0.1% NaCl, and 0.05% $MgSO_4$) were added to all flasks. To determine the extent of yeast growth, a direct microscopic count was made on a blood-counting chamber. Mold growth was determined by weighing the washed and dried mold mats.

TABLE 1

Growth of Saccharomyces cerevisiae in glucose and sucrose solutions

SUGAR	CONCENTRATION	START	YEAST CELLS PER MILLILITER (THOUSANDS OMITTED) TIME OF INCUBATION IN DAYS					
			1	2	4	6	8	10
	per cent							
Sucrose	30	300	3,100	6,500	34,000	25,000	25,000	25,000
Glucose	30	300	480	750	2,775	22,750	21,600	19,500
Glucose-Sucrose	30	300	675	950	28,750	25,000	25,000	25,000
Sucrose	40	300	420	625	12,750	22,500	22,500	22,500
Glucose	40	300	325	500	525	625	1,240	2,750
Glucose-Sucrose	40	300	450	800	4,850	6,750	9,100	12,500
Sucrose	50	300	150	275	650	850	2,750	4,500
Glucose	50	300	25	100	450	700	900	950
Glucose-Sucrose	50	300	50	225	545	450	740	1,125

RESULTS

Inhibiting effect of glucose and sucrose

Flasks containing glucose, sucrose, and equal part mixtures of both sugars in solution were inoculated with yeast cells and mold spores. These solutions also contained added mineral salts (as above) which enhanced the growth of both the yeast and the mold. A preliminary experiment using only the sugars and distilled water established the desirability of adding these essential minerals to the sugar solutions and this practice was followed throughout the remainder of the investigation.

All series consisted of 30, 40, and 50 per cent sirups by weight of each type of sugar. Direct counts were made of the yeast cells during a ten-day incubation period at 30°C. (86°C.). The results are shown in table 1.

Table 1 indicates that inhibition was more marked as the content of either sugar or the mixture of both sugars was increased. Yeast growth was heavier, in general, in all the sucrose sirups than in either the glucose or the glucose-sucrose solutions. The mixture of the two sugars generally inhibited growth

to a degree between that of either sugar alone except in a few isolated cases. At the end of 10 days the cells in the 30 per cent glucose, 30 and 40 per cent sucrose, and 30 per cent glucose-sucrose solutions were in the stationary growth phase. The cells in the other sugar concentrations were near the end of the logarithmic growth phase.

The mold flasks were incubated in the dark for six days at 30°C. (86°F.). As shown in table 2, glucose was found to exhibit a greater inhibitive action than sucrose at all concentrations used. A mixture of glucose and sucrose in sirups inhibited the growth of *Aspergillus niger* to an extent between that of solutions of similar concentrations of glucose and sucrose alone. The lowered pH after growth was due to the fact that mold utilizes sugar to produce acids.

TABLE 2

Growth of Aspergillus niger in glucose and sucrose solutions

SUGAR	CONCENTRATION	INITIAL pH	FINAL pH	DRIED MOLD WEIGHT
	per cent			*mg.*
Sucrose	30	6.0	2.2	451
Glucose	30	5 8	2.4	239
Glucose-Sucrose	30	5.8	2.3	406
Sucrose	40	6.1	2.3	335
Glucose	40	5.7	2.4	121
Glucose-Sucrose	40	5.8	2.4	275
Sucrose	50	6.0	2.3	198
Glucose	50	5.4	2.8	97
Glucose-Sucrose	50	5.8	2.6	139

The inoculation consisted of about 8000 mold spores.

Hydrogen-ion concentration

Sterile sugar solutions were adjusted to pH 5.6 ± 0.1 by the addition of 0.1N NaOH or 0.1N HCl and the experiments were repeated. Data of this experiment were considered superfluous and omitted. It was found that there was little or no difference in the results in both the yeast and the mold flasks. In other words, the slight difference in the initial pH between the sucrose and the glucose solutions was found to be of little importance.

Effect of heat

To determine the effect of pasteurization, flasks containing 50 ml. of sterile sucrose, glucose, and glucose-sucrose solutions were heated for 15 minutes in a water bath at 100°C. (212°F.). Inoculations of yeasts and mold spores were made in the cooled solutions as before and the cultures were incubated.

The heat apparently had some effect on the inhibiting action of the glucose at least. The ability of glucose to inhibit yeast growth was greatly increased. The effectiveness of sucrose on the repression of yeast growth was increased

only in the 40 per cent concentration. Except for the greater inhibition effect of heat on the glucose solutions, the data are similar to those presented for unheated solutions and are therefore omitted.

Mold growth was not affected to any considerable extent by the heated sugar solutions.

Inhibiting action of sugars in fruit sirups

The effect was determined of sucrose, glucose, and mixtures of the two sugars in apple, pineapple, and grapefruit juices on the growth of *Aspergillus niger* and *Saccharomyces cerevisiae*. The natural sugar content of filtered King David apple juice was increased to 40 per cent and 65 per cent by the addition of anhydrous glucose, sucrose, and a mixture of equal parts of both sugars. Commercially packed pineapple and grapefruit juices were prepared in a similar

TABLE 3

Growth of Saccharomyces cerevisiae in apple, pineapple, and grapefruit sirups

SUGAR	CONCENTRATION	YEAST CELLS PER MILLILITER (THOUSANDS OMITTED)			
		Start	7 days' incubation		
			Apple sirup	Pineapple sirup	Grapefruit sirup
	per cent				
Sucrose	40	400	20,000	8,750	7,380
Glucose	40	400	11,000	5,420	5,040
Glucose-Sucrose	40	400	14,750	7,200	6,190
Sucrose	65	400	475	No growth	No growth
Glucose-Sucrose	65	400	425	No growth	No growth
Control		400	20,000	13,100	12,900

manner. All sirups were pasteurized and cooled before inoculations were made. Because a solution of glucose will crystallize at high concentrations, it was considered impractical to use a fruit juice with 65 per cent sugar made by adding glucose alone. Apple juice had a pH of 3.5; pineapple juice, 3.3; and grapefruit juice, 3.1.

At the end of seven days' incubation the 40 per cent glucose and the 40 per cent mixtures of the two sugars in all cases showed a slightly greater inhibitive effect on the yeast than did the 40 per cent sucrose sirups (table 3). No growth was evident after seven days in either the grapefruit or the pineapple sirups containing 65 per cent sugar. The apple sirups at this high sugar concentration had an inhibiting effect also but displayed some slight growth of *Saccharomyces cerevisiae*. Compared with their respective controls, both the grapefruit and the pineapple sirups showed a marked restrictive influence in all sugar concentrations on the yeast growth.

Table 4 shows that an apple sirup containing 40 per cent glucose was more effective in the inhibition of mold than either the 40 per cent sucrose or the 40 per cent glucose-sucrose sirup. The 65 per cent sucrose solution permitted only a slight mold growth, 71 mg., but the flask containing the 65 per cent glucose-sucrose mixture had no mold growth at all. In comparison with the control with no added sugar, the 40 per cent sucrose solution and the 40 per cent mixture had little inhibiting effect on the mold growth but the 40 per cent glucose solution showed marked inhibition.

Mold growth was quite similar in both pineapple and grapefruit sirups. In each case *Aspergillus niger* developed about half the growth in 40 per cent glucose that was manifest in 40 per cent sucrose sirup. In pineapple sirup the dried weight was 412 mg. in the 40 per cent sucrose flask and 201 mg. in the 40 per cent glucose flask. Similarly, in the grapefruit sirup the weights were 409 mg. and 224 mg., respectively. In both sirups the 40 per cent glucose-sucrose mixtures developed a mold mat whose weight was between that of the mold developed in either sugar alone. As in the case of the yeast, no mold growth was observed in the 65 per cent sucrose and glucose-sucrose flasks.

TABLE 4

Growth of Aspergillus niger in apple, pineapple, and grapefruit sirups

SUGAR	CONCENTRATION	DRIED MOLD WEIGHT		
		Apple sirup	Pineapple sirup	Grape fruit sirup
	per cent	*mg.*	*mg.*	*mg.*
Sucrose	40	470	412	409
Glucose	40	259	201	224
Glucose-Sucrose	40	401	311	311
Sucrose	65	71	No growth	No growth
Glucose-Sucrose	65	No growth	No growth	No growth
Control		482	673	621

The inoculation consisted of about 6000 mold spores.

DISCUSSION

The results indicate that 40 and 50 per cent glucose solutions are more effective than sucrose solutions of the same concentrations in the repression of growth of both the mold and the yeast studied. The difference in inhibiting effect between the two sugars is probably based on the different osmotic pressures exerted by the sucrose and glucose. It would seem reasonable that this inhibition of the growth of spoilage agents would be of importance in the manufacture of fruit products. The sugar concentrations chosen in this investigation are commonly used in commercial practice. Crystallization difficulties preclude the use of sugar in concentrations above 65 per cent in fruit products.

SUMMARY

A study was made of the effect of 30, 40, and 50 per cent aqueous solutions of sucrose, glucose, and mixtures of these two sugars on the yeast *Saccharomyces cerevisiae* and the mold *Aspergillus niger*.

In equal concentrations glucose was markedly more inhibitory than sucrose to *Saccharomyces cerevisiae* and *Aspergillus niger*. In general, mixtures of equal parts of the two sugars inhibited growth to a degree intermediate to that of either sugar alone at the same concentration.

The ability of glucose to inhibit yeast growth was noticeably increased after the sugar solution had been heated for 15 minutes at 100°C. (212°F.) and cooled. Mold growth appeared to be little affected by heat treatment of either sugar.

In apple, pineapple, and grapefruit sirups 40 per cent glucose inhibited the growth of *Saccharomyces cerevisiae* slightly more than did 40 per cent sucrose. *Aspergillus niger* displayed about half the mold mat development in 40 per cent glucose sirups as in the 40 per cent sucrose solutions.

In almost all cases the equal part mixture of both sugars yielded results that were intermediate to those of either sugar alone used in the same concentration.

REFERENCES

BERKELEY, EARL OF, AND HORTLEY, E. J. 1906 Osmotic pressure of some concentrated aqueous solutions. Trans. Roy. Soc. (London), **206**, 481.

BUCHANAN, J. H. 1932 Corn sugar for beverages. Glass Packer, **11**, 357.

FELLERS, C. R., MILLER, J., AND ONSDORFF, T. 1937 Dextrose in the manufacture of fruit and vegetable products. Ind. Eng. Chem., **29**, 946–949.

HUNZIKER, O. F. 1935 Condensed milk and milk powder. 5th Ed. Pub. by author, Lagrange, Ill.

RAMSEY, R. J., TRACY, P. H., AND RUEHE, H. A. 1933 The use of corn sugar in the manufacture of sweetened condensed skim milk. J. Dairy Sci., **16**, 17–32.

URBAIN, O. M., AND MILLER, J. N. 1930 Relative merits of sucrose, dextrose, and levulose as used in the preservation of eggs by freezing. Ind. Eng. Chem., **22**, 355–356.

SELECTIVE ANTIBIOTIC ACTION OF VARIOUS SUBSTANCES OF MICROBIAL ORIGIN[1]

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INTRODUCTORY

Since Pasteur first demonstrated that certain microörganisms are able to exert antagonistic or antibiotic effects upon other organisms, an extensive literature has accumulated (Waksman, 1941). The presence of living organisms is often necessary for the phenomenon of antagonism to take place. In many cases, the antagonist was found to produce an active substance responsible for this action. The active agent has been isolated, purified, and crystallized only in very few instances. Pyocyanase was the first antagonistic substance to have thus been obtained (Emmerich and Low, 1899). Several others have been isolated recently.

Certain facts have now become recognized concerning the nature of the phenomenon produced by antagonistic microörganisms: 1. The various active substances isolated from the different organisms vary considerably in their chemical nature; 2. these substances are selective in their action upon various organisms, showing variation even as regards specific types or strains of the different groups of bacteria acted upon; 3. the substances vary in the mechanism of their action, some being primarily bacteriostatic, and others bactericidal but not bacteriolytic, whereas still others are both bactericidal and bacteriolytic; 4. the antagonistic capacity is widely distributed among microörganisms and is not limited to any one group of bacteria or fungi.

Active antibiotic agents have now been obtained from representative types of spore-forming bacteria, non-spore-forming bacteria, actinomycetes and fungi. Some of these agents have not yet been isolated in a pure state; however, they are well recognized, both chemically and biologically, and can be characterized by their specific properties. The following substances or preparations have received the greatest consideration: 1. pyocyanase, 2. pyocyanin, 3. gramicidin, 4. tyrocidine, 5. penicillin, 6. gliotoxin, 7. actinomycin, and 8. streptothricin. The first four are of bacterial origin, the next two are produced by fungi, and the last two by actinomycetes. To these may be added several other preparations, of which the exact chemical nature or mode of action is less known, namely, actinomycetin, prodigiosin, fluorescin, microbial-lysozyme, active substances obtained from species of *Aspergillus* and from various other fungi and bacteria. Among the antagonistic phenomena which are now well recognized but for which no active substance has as yet been demonstrated, one may men-

[1] Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Microbiology.

tion the action of certain strains of *Escherichia coli* upon other bacteria, of various yeasts and of many fungi (Nakhimovskaia, 1938, 1939; Waksman, 1941).

The origin, chemical nature and activities of the more important antagonistic substances of microbial origin thus far recognized are summarized in table 1. Because different methods have been used for testing the action of these substances upon microörganisms and because different test organisms, known to vary greatly in the degree of sensitivity, have been employed, it is difficult to compare the results obtained by the different investigators, who have first

TABLE 1

Summary of known chemical properties of antibiotic agents of microörganisms

PREPARATION	ORGANISMS	CHEMICAL NATURE	ORGANISMS ACTED UPON	HEAT STABILITY
Pyocyanase	*P. aeruginosa*	Lipoid	Gram-positive and gram-negative bacteria	Thermostable
Pyocyanin	*P. aeruginosa*	Pigment	Largely gram-positive bacteria	Thermostable
Tyrocidine	*B. brevis* (*Tyrothrix* species)	Polypeptide	Largely gram-positive bacteria	Thermostable
Gramicidin	*B. brevis* (*Tyrothrix* species)	Polypeptide	Largely gram-positive bacteria	Thermostable
Penicillin	*P. notatum*	Non-nitrogenous body	Various aerobic and anaerobic bacteria	Thermolabile
Gliotoxin	*Trichoderma*, *Gliocladium*	Sulfur—containing ring compound	Fungi and bacteria	Thermostable
Actinomycin	*A. antibioticus*	Polycyclic nitrogen compound	All bacteria and fungi	Thermostable
Actinomyces lysozyme	Various actinomycetes (*A. violaceus*)	Protein	Stated to be similar to lysozyme	Thermostable
Streptothricin	*A. lavendulae*	Organic base	Various gram-negative and gram-positive bacteria	Thermostable

isolated or tested these preparations. Further, the action of these substances has only seldom been compared with that of known chemical compounds having similar properties or producing similar action.

EXPERIMENTAL

This study was undertaken for the purpose of throwing light upon the relative activity of antibiotic substances of microbiological origin and of well defined chemical compounds, by the use of the same technique and the same test organisms. In the study of the bacteriostatic action of these preparations, two

gram-negative bacteria, two spore-forming and two non-spore-forming gram-positive bacteria, and two actinomycetes were used as test agents. These organisms were selected because of previously established variation in the degree of their sensitivity to the active substances. The two actinomycetes varied but little in their response; hence, only the results obtained with one of these are reported. In many cases, other test organisms, in addition to the above were also employed.

In view of the marked effect of the composition of the medium upon the activity of the substance, three media were used, namely, nutrient agar, nutrient broth, and brain-heart-infusion agar. The cultures were streaked over the solidified agar plate or inoculated into the tubes containing the broth. Incubation took place at 28° or 37°C., depending on the optimum conditions for the growth of the particular test organism, for 1 to 3 days. The active substances were dissolved in water, or, when required, in alcohol and diluted with water. Each active substance was added, in ten different concentrations, to 10 ml. portions of the test media.

In order to avoid complication that would result from the reporting of all the details thus obtained, only those concentrations are reported at which bacterial growth became inhibited. Complete inhibition of growth of the test organism by a given concentration of the bacteriostatic agent is emphasized by the use of a minus (−) sign. When no inhibition was obtained even with the highest concentration employed, a plus (+) sign is used. To illustrate this: 3 = partial inhibition of growth of the particular test organism by the addition of 3 milligrams of the substance to 10 ml. of agar or broth; 3− = complete inhibition of growth by 3 milligrams of the substance; 3+ = no inhibition at all with 3 milligrams of the substance, no higher concentration being used for making the particular test.

The following 14 preparations and chemical compounds were employed in these studies, nine of these being of microbiological and one of biological, although not microbiological, origin; four were well defined chemical compounds of non-biological origin.

1. *Actinomycin*, isolated from *Actinomyces antibioticus*, a crystalline substance (Waksman and Woodruff, 1941). The results reported in table 2 were calculated on a dry basis of purified actinomycin A.

2. *Streptothricin*, isolated from a strain of *Actinomyces lavendulae*. (Waksman and Woodruff, 1942). This was a highly purified preparation, although not in a crystalline state. A concentrated solution was used, containing, per 1 ml., 4 mg. of dry matter. The results are reported on the basis of dry material.

3. *Gramicidin*, first isolated from *Bacillus brevis* by Dubos (Dubos, 1939; Dubos and Hotchkiss, 1941).

4. *Tyrocidine* was also isolated by Dubos from *B. brevis* (Dubos, 1939; Dubos and Hotchkiss, 1941).

5. *Tyrothricin*, a mixture of substances 3 and 4, or the total active material obtained from *B. brevis*.

6. *Pyocyanase*, a crude preparation isolated from an active culture of *Pseudomonas aeruginosa*, by extraction with ether and concentration.

7. *Pyocyanin*, which is also produced by *P. aeruginosa*; however, for these studies, a synthetic preparation was used.

8. *Penicillin*, a highly purified preparation obtained from a culture of *Penicillium notatum*.

9. *Gliotoxin*, isolated from *Gliocladium fimbricatum* in a crystalline form.

10. *Lysozyme* or fresh egg-white. The results are reported on the basis of ml. of egg-white. The inclusion of this material is justified, because of the claims of Russian investigators that certain actinomycetes produce an active substance which is lysozyme-like in nature, and also because various bacteria are believed to be able to produce lysozyme.

11. *Tolu-p-quinone*, included in this study for two reasons: (a) actinomycin was found to contain a quinone group; (b) a comparison of the bacteriostatic action of several quinones has shown that tolu-p-quinone is a highly active bacteriostatic agent.

12. *Phenol*, the ordinary commercial product.

13. *Layryl sulfate* (Duponal M. E.) was included because of its marked detergent properties.

14. *Sulfanilamide*, included in this study because of its selective action against various bacteria, comparable to substances of microbial origin.

The results presented in table 2 show that the medium chosen for measuring bacteriostatic action of different preparations is of considerable importance. From 3 to 10 times as much actinomycin and streptothricin is necessary to inhibit the growth of the various test bacteria on brain-heart-infusion agar as on nutrient agar. This may be due to the presence in the brain-heart agar of specific inhibitors for these substances. Blood serum was found, in other experiments not reported here, to have no effect on the action of these substances; however, it is known to interfere with the action of tyrocidine. Nutrient broth proved much more favorable than agar media for demonstrating bacteriostasis of the products of *B. brevis*. This may be due to the low solubility of these substances in water and to their poor diffusion in agar.

The two gram-negative test bacteria were inhibited by streptothricin, pyocyanase, pyocyanin, gliotoxin, tolu-p-quinone, and high concentrations of phenol and sulfanilamide. The two aerobic spore-forming bacteria were inhibited by a number of the preparations, particularly by actinomycin and gliotoxin. It is of special interest to note that *Bacillus mycoides* (at least the particular strain employed in this test) was highly resistant, whereas *Bacillus subtilis* was sensitive to streptothricin, lysozyme, and sulfanilamide. Both organisms were resistant to gramicidin. *Micrococcus lysodeikticus* and *Sarcina lutea* were highly sensitive to actinomycin, gramicidin, tyrocidine, penicillin, gliotoxin and lysozyme. The *Actinomyces* sp. was sensitive to most of the substances when used in not too high dilutions.

Because of the claims that peptone interferes with the activity of the active substances, a synthetic medium was also used, namely Czapek's agar. The

TABLE 2

Bacteriostatic effects of agents of biological origin compared with pure chemical substances

Concentration of active substance, in milligrams, per 10 ml. agar or broth at which growth of the test organisms becomes inhibited; complete inhibition is designated by − sign; no inhibition with the given concentration is designated by + sign.

ACTIVE SUBSTANCE	TEST ORGANISM: *A. aerogenes*			*E. coli*			*B. mycoides*			*B. subtilis*			*M. lysodeikticus*			*S. lutea*		*Actinomyces* sp.	
TEST MEDIUM	NA*	NB	BHI	NA	NB	BHI	NA	NB	BHI	NA	NB	BHI	NA	NB	BHI	NA	BHI	NA	BHI
Actinomycin	3+	3	3+	3+	3−	3+	.001−	.001−	.01	.0003	.0003−	.01−	.0003	.0003−	.003−	.0003−	.003−	.03	.1−
Streptothricin†	.1	.3−	1	.03−	.03−	.3−	3+	3+	3+	.003	.01	.03	.03	.03−	.3	.03−	.3−	.03−	1−
Tyrothricin	30+	3+	3+	30+	3+	3+	.3−	.1	.3−	.3−	.03−	.3−	.003	.001−	.003−	.1	.1−	.3+	.3−
Gramicidin	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	.01	.0003−	.01−	.03	.1	3	3−
Tyrocidine	3+	3+	3+	3+	3+	3+	.1−	.03−	.1−	.1−	.01−	.1−	.03	.01−	.03	.03	.03	.1−	.1
Pyocyanase	1−	3+	3	1	3+	3	.3−	.3−	1−	.3−	.3−	1−	.1	.3−	1−	.3−	.3	1−	1−
Pyocyanin	1	3+	3	.3−	.3	1−	.1−	.1−	.3−	.03	.03	.3−	.1−	.03	.1−	.03−	.1−	.3−	.3−
Penicillin	3+	3+	3+	3+	3+	3+	3+	3+	3+	.01	.01	.01	.03−	.03−	—	.01	.01	1+	1+
Gliotoxin	1	3−	3−	1	1	1	.1	.03	.1−	.003	.03−	.01	.0001	.0003	.001	.001−	.0001−	.1−	.1−
Lysozyme‡	1+	1+	1+	1+	1+	1+	1+	1+	1+	.03	.03−	1−	.003−	.001−	.0003	.001	.0003	1−	.3
Tolu-p-quinone	3−	3−	3	3−	3−	3−	.3	1−	3−	.3	.3−	3−	.3	.1	1	.1	3−	1	3−
Phenol	30−	10	30−	30−	10	30−	30−	30−	30−	10−	10−	30−	10−	10−	10−	10	10−	10	10
Lauryl sulfate§	100+	10+	100+	100+	10+	100+	1−	1−	3−	.3	.03−	.3	1−	.3	3−	1−	3−	1−	3−
Sulfanilamide	10	10	10+	10	10	10+	10+	10+	10+	1	1	10	10	10+	10	10	1	10	10

* NA = nutrient agar; NB = nutrient broth; BHI = brain heart infusion agar.
† Crude preparation.
‡ Fresh egg white, in ml.
§ Duponal M.E.

TABLE 3

*Bacteriostatic action of various agents on a synthetic medium**

ACTIVE SUBSTANCE	TEST ORGANISM: *A. aerogenes*	*E. coli*	*B. mycoides*	*B. subtilis*	*S. lutea*	*Actinomyces* sp.
Actinomycin	2+	2+	.001	.0003	0.0003	.1
Tyrocidine	10+	10+	.03	.03	0.03	.03−
Gramicidin			10	10+	0.1	3+
Phenol	30−	10	10	3	10	30−
Sulfanilamide	10	10	10	.1−	1−	10

* Czapek's agar used; for *S. lutea*, 0.3 per cent asparagine replaced 0.2 per cent $NaNO_3$.

results obtained (table 3) tend to show that, for most organisms, the activity of sulfanilamide is greater on the synthetic medium than on the peptone-containing media. This is somewhat true also of tyrocidine and of gramicidin. Actinomycin and phenol, however, did not show any marked differences on the two types of media.

Considerable variation was found in the degree of sensitivity of the different test bacteria to the various bacteriostatic agents employed. One may definitely conclude from these results that no one organism can be used as a single test agent for comparing the bacteriostatic properties of different substances. If phenol is used as a standard, the ratio between the inhibiting concentration of the particular substance against a certain organism and that of phenol gives the relative bacteriostatic activity of the substance for the specific organism. One may thus calculate that the "bacteriostatic phenol coefficient" of actinomycin for *B. subtilis* is 33,000 units; streptothricin, 3,330 units; tyrothricin, 33; tyrocidine, 100; pyocyanase, 33; pyocyanin, 330; penicillin, 1,000; lysozyme, 330; gliotoxin, 3,300; toluquinone, 33; and sulfanilamide, 10. Using *S. lutea* as the test organism, the corresponding coefficient figures for the above substances become 33,000, 330, 100, 330, 33, 330, 1,000, 10,000, 10,000, 100, and 1.

In order to compare these results with those obtained by other investigators, it is sufficient to take sulfanilamide. In these tests, sulfanilamide gave a bacteriostatic action against the two cocci and *B. subtilis* of 1 to 10 mg. per 10 ml. agar, or in dilutions of 1:1,000 to 1:10,000. MacKay (1941) reported recently that sulfanilamide permitted the growth of *Staphylococcus aureus* and *B. subtilis*, after 24 hours, in concentration of 1:5,000, on various serum media.

When a crude preparation of penicillin was employed, in the form of the original culture medium, it was found to be also effective against gram-negative bacteria as well as certain gram-positive organisms not affected by the purified preparation. The concentrations of medium required to inhibit *E. coli* and *Aerobacter aerogenes* varied from 0.1 to 0.3 ml. per 10 ml. agar. *Brucella abortus* was inhibited by even lower concentrations. The action of the purified material is in conformity with the results obtained by the British investigators (Abraham, *et al.*, 1941). The importance of the composition of the medium, method of extraction, nature of test organism and of strain of antagonist employed, which have been found to be of great importance in establishing the activity of the active substance of antagonistic organisms (Waksman, 1941) are thus shown to hold particularly true of the active agents of *Penicillium notatum*.

The bacteriostatic action of several substances of microbial origin has been determined for several other gram-negative bacteria (table 4). Marked differences were again brought out in the degree of sensitivity of the same organism to different preparations; different species of the same genus show different degrees of sensitivity against the same active substance. A comparative examination of the effect of some of the other substances against *B. abortus* gave the following results, in milligrams per 10 ml. of nutrient agar: actinomycin, 0.1, streptothricin, 0.05, gramicidin, 1, pyocyanase, 0.1 — and gliotoxin, 0.01 —.

A detailed study of the bactericidal action of two substances of microbial origin, namely actinomycin and streptothricin, brought out that they behaved differently by this method as well. Actinomycin affected *E. coli* to a limited extent, unless used in relatively high concentrations, but it acted vigorously upon *B. subtilis* and *M. lysodeikticus*. Streptothricin, on the other hand, had a marked bactericidal action upon both gram-negative and gram-positive bacteria. One further difference in the selective bactericidal action of these two substances was observed, namely, actinomycin brought about the death of the cells without accompanying lysis, whereas streptothricin exerted a bactericidal action often accompanied by lysis, especially in case of micrococci.

Actinomycin has a slow bactericidal effect against bacteria. This is evident even with gram-negative bacteria which are ordinarily highly resistant to this substance. The results of an experiment using *E. coli* cells as the test organism may be reported here. *E. coli* was grown on nutrient agar. The cells were removed, washed, and then suspended in sterile tap water. One ml. of the cell

TABLE 4

Bacteriostatic action of actinomycin upon different species of Azotobacter

ORGANISM	CONCENTRATION OF ACTINOMYCIN, MG. PER 10 ML. AGAR		
	1.0	0.2	0.05
A. vinelandii	0	2	3
A. agile	1	3	3
A. agile	2	3	3
A. indicum	0	1	3
A. chroococcum	0	2	3
A. beijerinckii	1	3	3
Fresh soil culture	2	3	3

0 = no growth; 1–3 = growth.

suspension and varying amounts of an aqueous suspension of actinomycin were added to sterile test tubes, and the volume made to 10 ml. with sterile tap water. The tubes were incubated at 28°C. After 3, 7, 24, and 48 hours incubation, the numbers of surviving cells were determined by plating. The results were found (Waksman and Woodruff, 1940) to represent a monomolecular reaction similar to the action of chemical disinfectants. The results can be presented graphically by plotting the logarithms of the numbers of viable cells against the time of incubation. The first plating was made after 3 hours incubation. Since the cells in the control did not change in number during the experiment, as evidenced by the straight line on the graph, this line was extended to the 0 hour ordinate, and the extension indicated by a broken line. With the highest amount of actinomycin, all cells were dead after 48 hours incubation. Since all cells died at some time between 24 and 48 hours incubation, the graph line was extended in a smooth curve to meet the abscissa, the last portion of this line being broken to indicate the extension.

An examination of the graphs (fig. 1) reveals that 0.004 mg. of actinomycin per 10 ml. suspension of resting cells of *E. coli* was insufficient to bring about a significant reduction in cell numbers. Ten times that concentration, or 0.04 mg., proved slowly bactericidal and 0.4 mg. per 10 ml. proved markedly bactericidal. Both of these values are less than that required to inhibit *E. coli* growth (1–2 mg. per 10 ml. nutrient agar). This would seem to indicate that nutrient agar may contain some substance inhibitory to the action of actinomycin, or that the rapid multiplication of *E. coli* on nutrient agar is sufficient to overcome the slow bactericidal action of actinomycin, this action becoming evident only when cell multiplication is prevented.

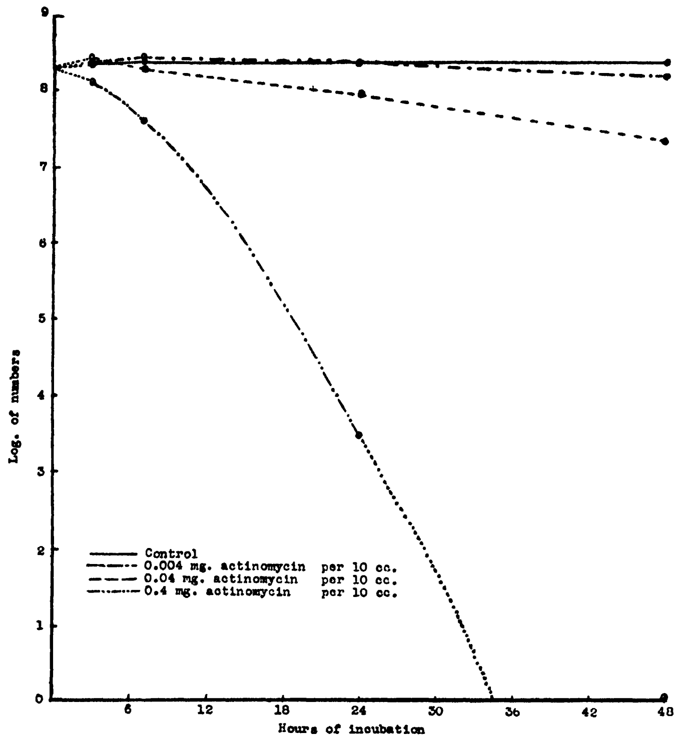

FIG. 1. DEATH RATE OF E. COLI CELLS IN BUFFER SOLUTION

Further comparative studies of the bactericidal action of different substances of microbial origin tended to emphasize their selective nature even more markedly. For example, gliotoxin, which exerted a fairly strong inhibiting effect upon *E. coli*, had only a limited bactericidal action, even in concentrations of 2 mg. per 10 ml. of broth culture. On another gram-negative organism, *Brucella abortus*, gliotoxin also exerted a lower bactericidal effect than actinomycin, crude penicillin, streptothricin and pyocyanase; the last two were by far most active in destroying this gram-negative pathogen. On the other hand, gliotoxin appeared to be more active bacteriostatically against certain bacteria than some of the other substances and preparations.

DISCUSSION

The recent interest in the antagonistic interrelationships of microörganisms has led to the preparation of several substances of microbial origin which have marked bacteriostatic and bactericidal properties. Some of these have been isolated in the form of pure chemical compounds, whereas others have so far been demonstrated only in the form of crude preparations containing the active principle. The methods employed by various investigators for measuring bacteriostasis vary considerably, both as to medium employed and as to test organisms used. No adequate comparison can, therefore, be made of the activity of the various antibiotic substances produced by different antagonistic microörganisms.

In this investigation, nine products of microbial origin, one other agent of biological origin and four synthetic chemical compounds have been tested by means of standard procedures for measuring bacteriostasis; seven test organisms and three different media were employed. Two of the substances, actinomycin and streptothricin, were of actinomyces (*A. antibioticus*, *A. lavendulae*) origin; three, tyrothricin, tyrocidine and gramicidin, were obtained from a spore-forming bacterium (*B. brevis*); two, pyocyanase and pyocyanin, from a non-spore forming gram-negative bacterium (*P. aeruginosa*); two, gliotoxin and penicillin, were of fungus origin (*Gliocladium fimbricatum* and *Penicillium notatum*). Egg white was used as the source of lysozyme, because an antagonistic agent obtained by certain investigators from different microörganisms was described as resembling lysozyme. For comparative purposes, phenol, tolu-p-quinone, lauryl sulfate, and sulfanilamide were also included.

The differences in the degree of sensitivity of the particular test bacteria to the various substances were most striking. Gramicidin was found to be most specific in its action, being limited to the cocci (*S. lutea* and *M. lysodeikticus*) and acting to a slight extent upon actinomycetes. Purified penicillin was next to it in specific action. Actinomycin, tyrothricin, tyrocidine and gliotoxin acted primarily upon the gram-positive organisms and actinomycetes, much less upon gram-negative bacteria. This is in contrast to the generalized, even if more limited action of phenol and tolu-p-quinone, which behave alike in regard to both gram-positive and gram-negative organisms. Pyocyanase, pyocyanin and the crude penicillin (*P. notatum* culture filtrate) were similar to the chemical compounds in their action, since they were found to be generally bacteriostatic over the wide range of the test organisms employed, no sharp division being obtained upon the basis of the gram stain. Streptothricin was unique in its action: the gram-positive spore-former *B. subtilis* was most sensitive but the other spore-former, *B. mycoides*, was not affected at all by this substance; the gram-negative *E. coli* was more sensitive to streptothricin than either *M. lysodeikticus* or *S. lutea*. Sulfanilamide proved to be only slightly bacteriostatic; however, it had a definite retarding effect upon the growth of the various organisms used in this study.

As a whole, the substances of microbial origin were found to be stronger bac-

teriostatic agents than the chemicals tested. This is based upon a comparison of the actual weights of the active materials. However, if the molecular weights had been used as a basis, the differences would have become even more magnified since the microbial agents are of considerably higher molecular weight than the chemical disinfectants.

It was found further that a high bacteriostatic action is not necessarily accompanied by a high bactericidal action. Gliotoxin, for example, was probably the most active bacteriostatic substance. However, its bactericidal properties were much lower than those of other preparations. Streptothricin, on the other hand, was highly active bacteriostatically and bactericidally against certain gram-negative bacteria. Certain gram-positive organisms, such as *B. mycoides*, usually very sensitive to the action of other compounds such as actinomycin, was highly resistant to streptothricin.

These results tend to disprove certain claims (Dubos and Hotchkiss, 1941) in regard to specific morphological differences in the behavior of bacteria in accordance with the gram stain. It is true that as a rule the gram-positive bacteria are more sensitive to most of the antibiotic compounds than the gram-negative organisms. But there are substances which act quite differently, showing marked variations within each group. One might just as well separate all spore-forming bacteria on the basis of the action of streptothricin, or separate the spore-forming gram-positive bacteria from the gram-positive cocci on the basis of their sensitivity of gramicidin.

It may also be noted here that some of the antibiotic substances have marked fungistatic and fungicidal properties. This is true especially of gliotoxin and actinomycin. These substances further vary in the degree of their toxicity to animals and in their action *in vivo*. The latter depends upon the solubility of the substances in water, their interaction with blood serum, their rate of excretion, absorption by special organs, etc.

In general, the following eight criteria must be carefully watched in comparing the selective action of antibiotic substances of microbial origin:

1. The test organism employed for measuring antagonistic action, namely, fungi vs. bacteria, gram-positive vs. gram-negative bacteria, one species vs. another species, one strain of the same species vs. another strain, etc.
2. Method of testing, including the composition of the medium, solid vs. liquid media, diffusion of active substance vs. suspension in medium.
3. Bacteriostatic vs. bactericidal action.
4. Mechanism of inhibition of bacterial growth by active substance.
5. Degree of purity of active substance.
6. Solubility of active substance, namely aqueous vs. alcoholic solution.
7. Stability of substance, as influenced by temperature, aeration, reaction.
8. Action *in vitro* vs. action *in vivo*.

SUMMARY

A comparative study has been made of the bacteriostatic and bactericidal properties of various known substances of microbial origin. Their action was

compared with that of egg-white lysozyme and different chemical agents commonly used as disinfectants. Considerable variation was obtained by the use of different test organisms and different media for testing. A number of different organisms and several media were employed for measuring the comparative bacteriostatic action of the different preparations. From three to ten times as much actinomycin or streptothricin was necessary to cause the same inhibition in brain-heart-infusion agar as in nutrient agar. Inhibition by the slightly soluble products of *Bacillus brevis* was more marked in nutrient broth than in the agar media.

Striking differences were found to exist in the selective action of these substances upon various bacteria. Gramicidin was most specific, acting primarily upon gram-positive micrococci. Actinomycin, tyrothricin, tyrocidine, purified penicillin, gliotoxin and the chemical detergent (lauryl sulfate) acted in low concentrations upon gram-positive bacteria and only to a limited extent upon gram-negative organisms. Pyocyanase, pyocyanin and crude penicillin were similar in their action over the whole range of the test organisms used. Streptothricin was unique in its action, being highly active against certain gram-negative bacteria and having no action against certain gram-positive organisms. Upon a weight basis, the substances of microbial origin were found to be much stronger bacteriostatic agents than the chemical antiseptics tested.

Marked differences were also obtained in the selective bactericidal action of the different preparations. Certain substances possessing high bacteriostatic properties were not necessarily also highly bactericidal.[2]

REFERENCES

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A., AND FLOREY, H. W. 1941 Further observations on penicillin. The Lancet, **241**, 177-189.

DUBOS, R. J. 1939 Bactericidal effect of an extract of a soil bacillus on Gram-positive cocci. Proc. Soc. Exptl. Biol. Med., **40**, 311-312; J. Exptl. Med., **70**, 1-17.

DUBOS, R. J. 1941 The significance of the structure of the bacterial cell in the problems of antisepsis and chemotherapy. Univ. Penn. Bicentennial Conference on Chemotherapy, p. 29-42.

DUBOS, R. J., AND HOTCHKISS, R. D. 1941 The production of bactericidal substances by aerobic sporulating bacilli. J. Exptl. Med., **73**, 629-640.

EMMERICH, R., AND LOW, O. 1899 Bakteriologische Enzyme als Ursache der erworbenen Immunität und die Heilung von Infektionskrankheiten durch dieselben. Z. Immunitäts., **31**, 1-65.

MACKAY, M. E. 1941 The bacteriostatic effect of disinfectants in human serum and citrated plasma. Brit. Med. J. No. 4193, 747-750.

NAKHIMOVSKAIA, M. I. 1938, 1939 Antagonism among bacteria. Microbiology (U.S. S.R.), **7**, 238-264; **8**, 1014-1032.

NOVOGRUDSKY, D. M. 1936 Antagonistic interrelations among microbes and biological methods of combating fungus diseases of cultivated plants. Adv. Biol. Sci., **5**, 509-536.

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WAKSMAN, S. A. 1941 Antagonistic relations of microorganisms. Bact. Revs., **5**, 231–291.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1940 Bacteriostatic and bactericidal substances produced by a soil Actinomyces. Proc. Soc. Exptl. Biol. Med., **45**, 609–614.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1941 Actinomyces antibioticus, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. J. Bact., **42**, 231–249.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1942 Streptothricin, a new selective bacteriostatic and bactericidal agent, particularly active against gram-negative bacteria. Proc. Soc. Exptl. Biol. Med., **49**, 207–210.

NOTES

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THE EFFECT OF BENZOIC ACID AND RELATED COMPOUNDS ON THE GROWTH OF CERTAIN MYCOBACTERIA

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In none of the bacteria for which p-aminobenzoic acid is essential does this compound act as a source of energy. On the other hand, related compounds, benzoic acid and hydroxybenzoic acids are readily oxidized by certain mycobacteria (Bernheim, 1940, 1942). The oxygen uptake of H37 and B_1 strains of the tubercle bacillus is increased in the presence of benzoic and o-hydroxybenzoic acids but not in the presence of the m- or p-hydroxybenzoic acids. A non-pathogenic species of mycobacteria obtained from Dr. Van Niel's collection oxidizes benzoic acid, m- and p-hydroxybenzoic acids but not o-hydroxybenzoic acid. It was therefore of interest to test the effect of these compounds on the growth of these bacteria.

Experiments were carried out first with the non-pathogenic species. This species will grow with 0.2 per cent glucose as the sole source of carbon in a salt mixture already described (1942). The water was doubly distilled from alkaline $KMnO_4$ and the glassware soaked in cleaning fluid for 48 hours. Upon replacing the glucose with either 0.2 per cent benzoic, or o-m- or p-hydroxybenzoic acids it was found that growth occurred in all the tubes except those containing the o-hydroxybenzoic acid. The bacteria can thus utilize as a sole source of carbon only those compounds that they are able to oxidize. The growth in the tubes containing m- or p-hydroxybenzoic acids was as rapid as that in the glucose controls and reached the same density. The growth in the tubes containing benzoic acid was slower at the beginning, but reached the same end point. This corresponds to the fact that in the oxidation of benzoic acid there is a latent period which is much shorter when m- or p-hydroxybenzoic acid is oxidized. Evidently the introduction of the first oxygen into the ring

is the limiting factor. Control experiments showed that these bacteria could not use bicarbonate as a source of carbon.

The H37 and B_1 strains of the tubercle bacillus were obtained from Saranac Lake. On veal-glycerol infusion broth the B_1 strain grows considerably more slowly than the H37. Addition of benzoic or o-hydroxybenzoic acid to this media in concentrations up to 5.0 mgm. per cent has no effect on the growth of either strain. Above this concentration the o-hydroxybenzoic acid inhibits growth whereas the benzoic acid up to a concentration of at least 15.0 mgm. per cent has no effect.

Similar results were obtained with Long's synthetic media and the H37 strain. The B_1 strain did not grow on this medium. The benzoic acids could not act as the sole source of carbon. Controls showed that the H37 strain grew almost as well in the absence of the asparagin as it did in its presence. At the end of 28 days, the average dry weight of five cultures grown on the complete media was 984 mgm, in the absence of asparagin it was 692 mgm. Washed suspensions of the H37 strain can oxidize and deaminate asparagin, indicating that it can utilize this compound if present. On the other hand the B_1 strain under the same conditions is unable to oxidize asparagin.

REFERENCES

BERNHEIM, F. 1940 The effect of various substances on the oxygen uptake of the tubercle bacillus. J. Bact., **41**, 387-395.

BERNHEIM, F. 1942 The oxidation of benzoic acid and related substances by certain mycobacteria. J. Biol. Chem., **143**, 383-389.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

KENTUCKY BRANCH

FIFTH SPRING MEETING, APRIL 18, 1942

FERMENTATION OF SOME SIMPLE CARBOHYDRATES BY MEMBERS OF THE PSEUDOMONAS GENUS. *Leon Stein, R. H. Weaver and M. Scherago,* Dept. of Bacteriology, University of Kentucky.

A comparative study of methods for the determination of carbohydrate utilization by members of the *Pseudomonas* genus has been made.

The results of these experiments make the following points evident:

1. The ordinary meat-extract sugar broth medium, with indicator, is not to be relied upon to show acid production by members of this genus.
2. A minimum buffered synthetic medium will reveal any acid production.
3. Since the results of the potentiometric determinations confirm those obtained in the synthetic medium, with indicator, such determinations do not seem to be necessary, at least in routine work.
4. Many strains of *Pseudomonas* utilize sugars without the production of any evident acid.
5. The use of a synthetic medium, without indicator, containing the carbohydrate as the only source of energy and of carbon, with growth as evidence of utilization, is the best routine method for the determination of carbohydrate utilization by members of the *Pseudomonas* genus.
6. Many species of *Pseudomonas* were found to produce carbon dioxide when Eldredge tubes were employed.
7. All of the 24 strains of *Pseudomonas* which were studied have been found to utilize glucose, the majority to utilize maltose and some to utilize sucrose and lactose.

THE PROBLEM OF THERMODURIC BACTERIA IN SHORT-TIME HIGH-TEMPERATURE PASTEURIZATION. *V. F. O'Daniel,* Ewing Von-Allmen Dairy Co., Louisville, Ky.

The isolation of thermoduric organisms from milk and a consideration of their thermal resistance indicates that the thermoduric problem is not to be overcome by increasing the temperature or time of pasteurization, but rather by the elimination of the organisms from the raw supply by educating the producers to use the proper sanitary practices.

Line-run tests at hourly intervals for three consecutive days showed that the pasteurizer was causing an average reduction in bacterial count of 89.1 per cent. After the sanitary conditions on the farms of 48 shippers (out of 350) whose milk had been found to pasteurize out with high counts had been improved, a similar study showed that the pasteurizer caused an average reduction in bacterial count of 98.5 per cent.

ENTRANCE OF NON-MOTILE BACTERIA AND CHEMICALS INTO WATER-SOAKED TOBACCO LEAVES. *Stephen Diachun, W. D. Valleau, and E. M. Johnson,* Kentucky Agricultural Experiment Station.

It was previously reported that water-soaking of tobacco leaves enables leafspot bacteria to enter the leaves, presumably through water channels occurring in the stomata from the outside to the inside of the leaf. The object of the study reported here was to determine whether the bacteria enter the leaves by means of their own motility, or are carried in by action of some outside force.

Non-motile bacteria (*Staphylococcus aureus*) were placed on water-soaked tissue and non-water-soaked tissue of the same leaf. After the water-soaking disappeared (within 30 minutes) the leaf surface was sterilized with $HgCl_2$; representative portions of the leaf were cut out, crushed, and mixed with agar in petri plates. Within a few days thousands of colonies of *S. aureus* developed on each plate prepared from water-soaked tissue; no colonies were present on plates prepared from non-water-soaked similarly inoculated tissue. This

test shows that swimming is not necessary for bacterial invasion of leaves.

India ink entered water-soaked leaf tissue rapidly, producing a blackening that could not be washed off; ink did not enter non-water-soaked tissue.

Solutions of $HgCl_2$ and $CuSO_4$, and Bordeaux mixture entered water-soaked leaf tissue rapidly, producing necrosis. This suggests the possibility that naturally induced water-soaking may play a part in the occurrence of spray injury.

SOME EXPERIMENTS ON THE EXCRETION OF NITROGEN COMPOUNDS FROM LEGUME ROOTS. *Hugh G. Myers*, Kentucky Agricultural Experiment Station.

Results of greenhouse and laboratory experiments at the Kentucky Station on the excretion of nitrogen compounds by legume roots as a means of N transfer from a legume to an associated nonlegume, were similar in general to the published results of other workers. Excretion occurred in less than three per cent of associated combinations of vetch and rye; and no excretion was obtained with mixtures of vetch and ryegrass, red clover and orchard grass, or alfalfa and bromegrass. Varying such factors as day length, light intensity, temperature, level of potassium in the substrate, and the strain of *Rhizobium* failed to bring about conditions consistently favorable to excretion. This mode of transfer of nitrogen from legume to nonlegume is of course different from that which occurs in disintegration and decomposition of legume roots.

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

MISSOURI VALLEY BRANCH

UNIVERSITY OF NEBRASKA, LINCOLN, MAY 2, 1942

COMPARATIVE STUDY OF MEDIA USED FOR THE ISOLATION OF ORAL STREPTOCOCCI. *K. D. Rose and C. E. Georgi*, Department of Bacteriology, University of Nebraska, Lincoln, Neb.

A series of media designed for the selective isolation of oral streptococci have been tested in the laboratory on a comparative basis with micro-organisms likely to be found on eating utensils or in dishwater. The results indicate that media containing sodium azide, sodium azide and blood, or potassium tellurite are less inhibitory to organisms other than streptococci than a medium containing potassium tellurite and crystal violet, but the latter is also quite inhibitory to "typical" *Streptococcus salivarius*. A medium containing sodium azide and potassium tellurite inhibits all contaminants but *Staphylococcus aureus* and a *Proteus* species, the latter growing only in small discrete colonies. "Typical" *Streptococcus salivarius* grows without inhibition on this medium when the inoculum is sufficiently large to initiate growth. Experiments with serial dilutions of this organism indicate that the inoculum needed is greater than that normally occurring on restaurant glasses. A primary selective enrichment culture is probably necessary to assure 100% isolations in sanitary studies.

THE INFLUENCE OF MICROORGANISMS AND BIOLOGICAL PRODUCTS ON THE PHYSICAL PROPERTIES OF A LOESSIAL SUBSOIL. *T. M. McCalla*, Soil Conservation Service and Nebraska Experiment Station, Lincoln, Neb.

A loessial subsoil containing 0.2 percent organic matter and 16 percent inorganic colloid was used to determine the magnitude of the binding effect of microorganisms and biological products on the soil particles. Falling water drops were used to determine the energy required to destroy the structure formed in the presence of organic matter of microorganisms as compared with the original soil.

The incorporation of organic matter into the loessial subsoil and the use of plant residue cover resulted in a high intake of water. When the untreated soil was used with a cover, the water intake was much lower. Water intake into the soil in which the growth of microorganisms was stimulated by sucrose treatment was high, even without a cover to break the impact of the falling water drops. The thousands of

fungus filaments bound the soil together into stable masses.

THE HEAT RESISTANCE OF MIXED CULTURES OF STREPTOCOCCUS THERMOPHILUS AND CERTAIN CASEOLYTIC BACTERIA. *H. J. Peppler*, Department of Bacteriology, Kansas State College of Agriculture and Applied Science, Manhattan, Kansas.

The activity of *Streptococcus liquefaciens*, *Pseudomonas aeruginosa*, *Proteus ammoniae*, and other caseolytic bacteria, in a skimmilk medium with *Streptococcus thermophilus* has been shown to stimulate the growth of *S. thermophilus* and to increase the activity of subcultures grown at 48°C. following heating at 65°C. for 30 minutes. The degree of stimulation and increase in heat resistance of *S. thermophilus* varies with the number of caseolytic bacteria added. The heat resistance of some mixed cultures was equivalent to that of pure cultures of *S. thermophilus* grown in milk media enriched with different commercial peptones. The activity of heat-treated subcultures of mixed cultures decreased sharply after the first culture generation as a result of the gradual suppression of the caseolytic associate.

Enrichment of skimmilk with small amounts of heat-killed whey cultures of the caseolytic bacteria stimulated the acid production and increased the heat resistance of pure cultures of *S. thermophilus* to the same extent observed with corresponding mixed cultures.

The increased heat resistance of the lactic acid organisms is probably related to the availability of nitrogen in the culture medium before heat treatment. Alkali-treated peptones added to the skimmilk medium for *S. thermophilus* possessed the same degree of stimulation exhibited by untreated preparations. Various accessory substances, such as calcium pantothenate, l-ascorbic acid, riboflavin, thiamin, and niacin added separately or in different combinations to the culture medium, did not influence the heat resistance of *S. thermophilus*.

When grown in skimmilk for 24 hours at 32°C. and then heated at 55° or 58°C., all caseolytic bacteria, except *P. aeruginosa* and *Achromobacter lipolyticum*, survived after 60 minutes. At 55°C. *P. aeruginosa* and *A. lipolyticum* were killed in milk within 25 minutes; at 58°C. both organisms failed to grow in milk after heating for 10 minutes.

A STUDY OF CERTAIN FACTORS WHICH INFLUENCE THE APPARENT HEAT RESISTANCE OF BACTERIA. *F. E. Nelson*, Kansas Agricultural Experiment Station.

The effects of different plating media and of variations in temperature and time of incubation upon the quantitative enumeration of heat-treated bacteria were studied. *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus durans*, *Streptococcus liquefaciens* and *Streptococcus zymogenes* were used.

Within fairly wide limits of variation the factors studied had little or no effect upon the counts of unheated control cultures. Beef-infusion agar usually gave higher counts of heated bacteria than were obtained from three other media. For some lactic streptococci the new standard milk agar was very superior to ordinary nutrient agar. Incubation at 32°C. usually resulted in counts higher than at 21°, 28°, 37°, or 42°C., and 96-hour counts were appreciably higher than 48-hour counts, especially at the lower temperatures of incubation. Heated bacteria were much more sensitive to the pH of the recovery medium than were the control organisms. Addition of thioglycollic acid in amounts as small as 0.00021 percent usually resulted in considerable increases in plate count of the heat-treated bacteria, while larger amounts had a still greater effect up to a limit which was a function of medium and organism. Cysteine had a similar effect.

By suitable plating procedures considerable differences can be caused in the apparent number of survivors among heated bacteria.

GROWTH OF NON-SPORULATING ANAEROBIC BACTERIA OF INTESTINAL ORIGIN IN SYNTHETIC MEDIA. II. Essential Components and Additional Growth Stimulants. *Don H. Larsen and Keith H. Lewis*, Department of Bacteriology, University of Nebraska, Lincoln.

Elimination of unnecessary constituents from a previously developed synthetic

medium consisting of 10 mineral salts, glucose, sodium lactate, glycerol, 17 amino acids and 14 growth factors has been attempted. Duplicate cultures of 15 or more representative strains of non-sporulating anaerobic bacteria were employed in all experiments.

A combination of K_2HPO_4, KH_2PO_4, NaCl and $MgSO_4$ gave nearly as satisfactory growth alone as with the other six salts. Omission of sodium lactate and glycerol did not lessen growth in any instance and occasionally improved it. Elimination of amino acids either singly or in combination reduced the growth of the group as a whole. Individual strains could, however, develop in simplified mixtures of the amino acids. Of the 14 original growth factors all except pyruvic acid, pantothenic acid, nicotinic acid, pyridoxine and riboflavin were omitted without extensively changing the properties of the medium. Biotin, folic acid, adenylic acid, choline and p-amino benzoic acid, not included in the original 14 growth factors, were also tested. Only p-amino benzoic acid was active in the concentrations used. Addition of tryptone, liver extract or tomato juice to the synthetic medium improved growth, thus indicating that additional unknown factors are needed.

BACTERIUM NECROPHORUS SEPTICEMIA IN MAN. *Victor B. Buhler, Clark W. Seely, and Dorothy D. Dixon*, General Hospital, Kansas City, Mo.

Two cases of *Bacterium necrophorus* infection with septicemia occurring in the human being are recorded in this paper. It is the opinion of the authors that *B. necrophorus* invasion in man is probably not such a rare occurrence as is commonly thought. They feel that more active investigation of common infections by anaerobic methods may reveal this organism in a larger number of cases.

B. necrophorus has long been fairly well known to veterinary medicine as the causative agent in gangrenous stomatitis, lip, and leg ulcerations, and abscesses of the liver in horses, cattle, sheep, and numerous other domestic and wild animals.

Human infections caused by *B. necrophorus* are reviewed describing involvement of many organs including skin, lungs, nasopharynx, gastro intestinal tract, and genitourinary system. Only one proved case of septicemia is previously recorded.

The two cases presented are reviewed in full giving case histories, hospital course, laboratory, and autopsy material.

The source of *B. necrophorus* was thought to be from a peri-appendicitis in one case and from a perforating wound of the perineum in the other.

From the bacteriological aspect the organism is described morphologically and culturally. The media and methods used for isolation and identification are given in detail, with an accompanying comparative chart of *Bacterium necrophorus* and closely related organisms. The bibliography contains 31 references.

GALACTURONIC ACID, A CONSTITUENT OF A BACTERIAL GUM. *C. E. Georgi, W. E. Militzer, K. B. McCall, and D. A. Bixler*, Departments of Bacteriology and Chemistry, University of Nebraska, Lincoln, Nebraska.

An unidentified bacterium produces large quantities of a polysaccharide gum when grown on an agar medium containing sucrose as the carbon source, KNO_3 as the nitrogen source and in addition, K_2HPO_4. Mass cultures were grown in Kolle flasks. Cells were removed by centrifuging at 45,000 r.p.m. after the gum had been greatly diluted in a weak aqueous solution of Na_2OO_3 designed to reduce the viscosity so as to facilitate precipitation of the cells. Water was removed by heating to 50°C. at 15 mm. and the gum then precipitated with absolute EtOH. After filtering and drying, the gum was hydrolyzed with 2.5% H_2SO_4 and precipitated as the barium salt. Analysis of the latter indicated $Ba^{=}$ 26–27% which is the theoretical value for a hexose. Oxidation of the barium salt with bromine water yielded mucic acid. The orcinol test applied to the gum itself resulted in the detection of furfural. These qualitative tests point to the presence of galacturonic acid as one of the constituents of the polysaccharide gum.

BACTERIOLOGICAL STUDY OF MACHINE AND HANDWASHED DISHES AT HASKELL INSTITUTE. *Harold G. Nelson*, Dept. of

Bacteriology, Univ. of Kansas, Lawrence.

This study consisted of three examinations of the dishes from the dining halls, the cafe, and hospital at Haskell Institute, which is an Indian school located at Lawrence, Kansas.

The enrollment at the school is approximately 725 students. The hospital is a forty-bed institution. The dishes are washed by hand at the cafe and hospital while they are washed by machines at the dining halls.

The first samples were obtained February 16, 1942. The swab technic of Fellers, Levine and Harvey was employed. This technic was modified by streaking onto blood agar and proteose peptone III agar and inoculating lactose broth. The results were as follows: Machine washed, 8 organisms per article; Hand washed, 27 organisms per article.

Loeffler's tubes and lactose tubes were negative.

Samples were again obtained on the 24th of February and run out as above. The results were as follows: Machine washed, 13 organisms per article; Hand washed, 14 organisms per article.

Loeffler's tubes and lactose tubes were again negative.

The Kansas State Board of Health Method was employed on the third samples obtained February 27, 1942. This method is similar to the above method except that ten articles are swabbed rather than one. Blood agar and lactose broth are also included. The results were as follows: Machine washed, 31 organisms per article; Hand washed, 52 organisms per article.

Since alpha streptococci were found on the hospital and cafe samples, it was recommended that the cups and glasses in these places be rinsed in a chlorine solution.

The counts in all cases were quite low; however, the machine washed dishes yielded a lower count than did the hand washed dishes.

Preliminary Report on a Survey to Determine the Bacteriological Condition of Utensils in the Service of Food. *Evan Wright, Flora Acton and Charles A. Hunter*, Kansas State Board of Health, Topeka.

Utilization of Twenty-one Proprietary Peptones by Representative Aerobic and Facultative Bacteria. *Margaret McMaster and Carl E. Georgi*, Dept. of Bacteriology, Lincoln, Nebraska.

The growth of representative aerobic and facultative bacteria was followed qualitatively and quantitatively on twenty-one commercial peptones.

From the quantitative studies, it was observed that peptones vary a great deal in their ability to support the growth of the test organisms. Using peptones in concentrations of 0.1%, 0.01%, and 0.001% in semi-solid glucose peptone agar, it was found that four peptones supported growth vigorously in these low concentrations.

The peptones were compared qualitatively as to their effectiveness in the detection of acetyl-methyl-carbinol, the production of indole and of hydrogen sulfide.

All the peptones tested, but one, contained sufficient compounds possessing a guanidine nucleus to give good tests for acetyl-methyl-carbinol when the latter was produced by the bacteria.

Several peptones, five in number, were found to yield superior tests for the production of indole. Three did not contain enough tryptophane to give any positive tests with the organisms studied.

Of the three indicator ions (iron, lead, and bismuth) used for the detection of hydrogen-sulfide production, bismuth proved to be the most satisfactory, when used with the various peptones. Four peptones were found which yielded very good results with bismuth as the indicator. It was also found that the indicator used for the detection of hydrogen sulfide must be taken into account when a suitable peptone is being chosen. Three were not suitable for the demonstration of hydrogen-sulfide production with any of the indicator ions.

The Composition of Culture Media as It Affects the Fermentation Characteristics of Corynebacterium. *Harle Barrett, Flora Acton and Charles A. Hunter*, Kansas State Board of Health, Topeka, Kansas.

A considerable number of nose and throat cultures from a selected group of individuals revealed the presence of *Corynebacterium*.

These were isolated by using tellurite blood agar and studied.

Fermentation media were prepared by combining various fermentation bases and indicators. The fermentation of sucrose, glucose, and dextrin seemed to depend upon the composition of the culture media and the indicator used.

THE EFFECT OF GRAMICIDIN AND TYROCIDINE ON VARIOUS BACTERIA. *Cora M. Downs*, University of Kansas, Lawrence.

In a series of articles Dubos and Hotchkiss and Dubos have described the isolation and identification of two bactericidal agents: gramicidin and tyrocidine, the first active chiefly against gram-positive organisms, the second active against both gram-positive and gram-negative but more active against the latter. Downs has studied the effect of these substances on gonococci and meningococci. In the present study a comparison was made of the bactericidal substances in regard to their activity on various organisms. The substances were diluted in 5 per cent glucose to the desired concentration mixed with an equal volume of the organism suspended in glucose, incubated at 37°C. for three hours and cultured in suitable media. The following amounts of gramicidin and tyrocidine were found to be lethal for the organisms named: Pneumococci G* 0.01 g, T† 1 g: Hemolytic streptococci G 2 g, T 5 g, *Staphylococcus aureus* G 10 g, T 50 g, Meningococci G 5 g, T 1 g; Gonococci G 1 g; T .01 g; *Pasteurella avicida* T 500 g; *Pasteurella tularensis* virulent G 250 g, T 100 g; avirulent G 25 g, T 25 g; *Shigella dysenteriae* T 50 g; *Escherichia coli* T 500 g; *Salmonella schottmülleri* T 250 g; *Eberthella typhosa* 500 g.

Where no figures are given for gramicidin, 500 gs did not kill the organisms. Smaller amounts of the substances were bacteriostatic but not bactericidal. When the substances are used in amounts which are bactericidal the action is evident within the first hour as determined by plate counts. When bacteriostatic amounts are used there is a progressive decrease in numbers until after the fifth or sixth hours after which there is a gradual increase.

* Gramicidin in micrograms.

† Tyrocidine in micrograms.

STUDIES ON THE REAGIN CONTENT OF NORMAL AND SYPHILITIC RABBITS. *Noble P. Sherwood and Carolyn Collins*, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

The Kahn Verification test as well as other serological tests was used in the study of the blood serum of eighty-nine normal, adult, male rabbits. Fifteen of these were used as uninoculated normal controls and seventy-four of the rabbits were inoculated intratesticularly with *Treponema pallidum*. Darkfield examinations were made at various intervals of time to determine the presence or absence of spirochetes. The shortest interval of time elapsing between infection and darkfield examination was five days. The results of these examinations were positive.

The maximum titer of reagin in uninoculated animals or in animals before inoculation was three Kahn units. The minimum was no Kahn units. Fluctuations between completely negative and detectable amounts of reagin were observed in all normal controls. Following inoculation with *T. pallidum* there would develop, usually within two or three weeks, a marked increase in reagin. This might or might not be, in the beginning, of the syphilitic type according to Kahn's Verification test, but as infection progressed, this syphilitic type of reaction predominated although fluctuations occurred; for some weeks the general biological type of reaction would replace the syphilitic type.

Reagin titers of as much as thirty Kahn units were commonly reached during infection. After a variable number of weeks the reagin titer would drop, gradually, to within normal limits in most of the infected rabbits that we were able to study for a long period of time.

During infection there was not only variation in the kind of reaction but there was variation in amount of reagin from week to week. Some rabbits were studied as long as fifty-one weeks. In others the period of observation has been limited to ten weeks.

1941 KANSAS INTRASTATE EVALUATION STUDY ON THE PERFORMANCE OF SERODIAGNOSTIC TESTS FOR SYPHILIS. *Charles A. Hunter, and Frank Victor*, Public

Health Laboratories, Kansas State Board of Health.

Two-hundred-and-twenty-five blood specimens, of which 117 were from syphilitics and 108 were from presumably non-syphilitics, were sent to 31 laboratories that voluntarily entered the evaluation study. The U. S. Venereal Disease Research Laboratory also received a set of the specimens.

The State Laboratory served as the control laboratory.

The results were as follows:

Kahn Standard

Number of Laboratories entered........	25
Number of laboratories low in sensitivity	10
Number of laboratories low in specificity	7
Number of laboratories low in both.....	1
Total number of laboratories failing to meet standards.......	17

Complement Fixation

Number laboratories entered.	20
Number laboratories low in sensitivity. .	3
Number laboratories low in specificity...	6
Total number laboratories failed.......	9

Kline Diagnostic

Number of laboratories entered.	6
Number of laboratories low in sensitivity	0
Number of laboratories low in specificity.	6
Total number laboratories failed..	6

An Attempt to Demonstrate a Virus as the Cause of Mastitis in Cattle. *L. D. Bushnell*, Kansas State College, Manhattan.

In 1939, Broadhurst, Cameron and McLean reported on a filterable virus as the probable cause of mastitis in cows.

In our investigations, milk was obtained from cows with typical clinical mastitis and an attempt made to demonstrate a virus on the chorioallantois of the developing embryo of the hen's egg.

The cellular sediment was stained by several methods and various bodies which may have been inclusion bodies were demonstrated.

The milk was filtered and eggs of 10 to 12 days incubation were inoculated with from 0.1 ml. to 0.5 ml. In no instance could virus lesions be demonstrated.

Samples of milk treated with 50% glycerol, 1:1000 merthiolate, 1:1000 phenol, 1:1000 crystal violet finally became free of bacteria and were inoculated on the egg membranes. In a few instances there was a thickening or clouding of the membrane. Sections of these membranes were transferred to other eggs but we were unable to cultivate a virus by this means.

The conclusion reached was that we were unable to demonstrate a virus for mastitis by this means.

Bacterial Invasion of the Chorio-allantoic Membrane. *Cornelia M. Downs and Seymour S. Kalter*, Department of Bacteriology, University of Kansas, Lawrence, Kansas

This paper represents a preliminary report involving certain immunological aspects of bacterial infection of the chorio-allantoic membrane of chick embryos. The technic employed is essentially that of Goodpasture, *et al.*, and in all cases so far described the pathology of the infection of these membranes corresponds to that described by Goodpasture.

Studies thus far have been made with *Staphylococcus aureus* and *Neisseria meningitidis*.

Immunological studies so far have been limited to the use of immune sera placed directly upon the membrane immediately before adding the infecting dose. Immune rabbit serum against staphylococcus with an agglutinating titer of 1:640 was used. A commercial polyvalent horse antiserum was used for protection against the meningococci, with an agglutinating titer of 1:320. Our controls consisted of normal rabbit serum, in the case of the staphylococci, and normal horse serum for the meningococci, corresponding to the immune serum used.

There was no significant protection obtained by immune serum against staphylococci. The staphylococcus was recovered from the membrane after twelve hours. An exudate appeared at this time, followed by ulceration of the membrane. The exudate involved the mesoderm also. The organism grew extracellularly and there was no intracellular invasion.

When meningococci were used, the anti-

serum protected the embryo which survived what was a lethal dose of cocci in untreated chicks. In the untreated embryos only one lived 96 hours, with the majority dying before 72 hours. On both the protected and unprotected membranes, no exudate was noticed until 18 hours at which time the organism was recoverable from small pieces of the membrane. Grossly there is a thickening of the membrane and slight hemorrhagic areas were noted on the untreated membranes.

DEMONSTRATION OF THE TITRATION OF VARIOUS VIRUSES ON CHORIO-ALLANTOIC MEMBRANES. *Jean Rubbra, Carolyn Collins and Seymour Kalter*, University of Kansas, Lawrence.

JOINT MEETING, NEW JERSEY AND NEW YORK BRANCHES

PRINCETON, N. J., MAY 16, 1942

THE EFFECT OF CERTAIN CARCINOGENIC HYDROCARBONS ON THE GROWTH RATES OF E. COLI AND S. AUREUS. *G. David Novelli*, Department of Bacteriology, Rutgers University.

The effect of methylcholanthrene; 1,2,5,6,dibenzanthracene; 3-4-benzpyrene and 1,2-benzanthracene on the growth rate of *Escherichia coli* and *Staphylococcus aureus* was studied by making growth curves of the organisms in the presence of the hydrocarbons as compared with a standard control.

Both methylcholanthrene and 1,2,5,6-dibenzanthracene stimulated the growth rate of *E. coli* and *S. aureus* in the concentrations employed. Both of these compounds were more effective on *S. aureus* than on *E. coli*. Methylcholanthrene caused an increase in maximum numbers of 36% with *S. aureus* as compared to a 25% increase with *E. coli*. Dibenzanthracene caused an increase of 17% with *E. coli* as compared to a 19% increase with *S. aureus*. 1,2-Benzanthracene had no effect on *S. aureus*. It proved, however, to be slightly inhibitory to the *E. coli* culture. This inhibition did not occur until after the maximum numbers had been attained.

The effect of 3,4-benzpyrene was found to vary both with the concentration of the compound and with the size of the inoculum. The greatest effect on *E. coli* was found to occur when a small inoculum was used. In high concentrations and using a large inoculum the effect of this compound was seemingly inhibitory. At a concentration of 30 milligrams %, a concentration at which this compound was slightly inhibitory to *E. coli*, it caused a 52% inhibition of the *S. aureus* culture, suggesting that these compounds behave differently with different organisms.

REMARKS CONCERNING SOME RECENT DEVELOPMENTS IN BACTERIAL METABOLISM. *Dr. J. H. Quastel*, Rothamsted Experimental Station, England.

THE EFFECTS OF DETERGENTS ON PROTEINS, VIRUSES, AND BACTERIA. *Dr. M. L. Anson*, The Rockefeller Institute, Princeton.

OBSERVATIONS ON THE ANTI-BACTERIAL ACTION OF SURFACE ACTIVE CATIONS. *E. I. Valko and A. S. DuBois*, Onyx Oil and Chemical Company, Jersey City, New Jersey.

The bactericidal effect of a surface active cation, e.g., N-n-dodecyl-N′-ethyl-benzotriazolium ion is greatly diminished when other surface active cations which are less toxic to the bacteria, e.g., N-n-hexadexyl- or N-n-octadecyl-N′-ethyl-benzotriazolium ion, are present. The phenomenon indicates that, either on the surface of, or in the bacteria there are certain spaces available for the surface active cations. If these spaces are occupied by harmless cations the bacteria are protected against the toxic cations, provided the harmless cations are more firmly attached to the bacteria than the toxic ones.

The concentration of surface active cation which is determined by the F.D.A. method as killing, is not always lethal to bacteria. By detoxication with surface active anions the bacteria which were treated with the "killing" concentration can recover their

ability to growth. E.g., *Staphylococcus aureus* treated for 5 min. with alkyl-dimethyl-benzylammonium chloride of a concentration corresponding to five hundred per cent of the "killing" concentration can be revived by a 5 min. treatment with an equivalent amount of sodium lorol sulfate.

Chemical, Physical and Immunological Properties of Egg Albumin Denatured in Various Ways. *C. F. MacPherson, M. Heidelberger, and D. Moore*, Departments of Medicine and Anatomy, College of Physicians and Surgeons, Columbia University, New York City.

Calculations of the particle weights of purified acid, alkali and heat denatured egg albumins from sedimentation and diffusion measurements show that these substances are aggregates. The size of the aggregates depends on the type of product and on the extent to which it has been exposed to the aggregating influences of salt or long standing in the flocked state.

The viscosities of these products decrease from several months after preparation, and evidence from viscosity and diffusion data has been given to show that the shape of the aggregate resembles that of a disk.

The mobilities of acid and heat denatured egg albumin are the same as that of the native protein. The mobility of alkali denatured egg albumin is lower.

The different types of products have been found to exhibit small quantitative immunological differences.

Epidemic Kerato-Conjunctivitis. *Dr. Murray Sanders*, Department of Ophthalmology, College of Physicians and Surgeons, New York.

THE MICROBIAL FLORA OF THE ROCKY MOUNTAIN WOOD TICK, DERMACENTOR ANDERSONI STILES

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This report is concerned with the results of a study of the microbial flora of 2502 adult specimens of the Rocky Mountain wood tick, *Dermacentor andersoni*, obtained from various sources. The accumulated data suggest that the bacterial flora of this tick is largely fortuitous, consisting chiefly of adventitious organisms acquired while feeding on its host.

MATERIALS AND METHODS

Part of the ticks tested were from a laboratory strain initiated in 1934 and reared subsequently through several generations, and part were collected from nature in western Montana. There were 2016 unfed specimens and 486 that had recently received a blood meal. One hundred and forty-five lots were tested. A few of the lots contained numerous ticks, some only two; the remainder averaged 10 ticks each. In addition, a considerable number of ticks were dissected and the various organs examined separately.

Each lot was prepared for examination by first immersing the ticks in tincture of merthiolate for 4 to 5 hours, then rinsing them thoroughly with 5 changes of sterile saline (0.5 ml. of the last wash was cultured), and finally by triturating the ticks (except those dissected) in sterile saline. The resulting suspension was then examined for bacteria, yeasts, molds, rickettsiae, and motile protozoa by the techniques outlined below.

BACTERIA

Techniques

The triturated tick suspension was streaked on glucose beef-infusion agar, North's gelatin chocolate blood agar, and cystine heart agar plates. Tubes of Noguchi's leptospira medium were also frequently inoculated. As a routine test for anaerobes, deep tubes of sodium-thioglycollate broth containing minced pork brain were inoculated and the surface of the broth covered with a layer of vaseline. Occasionally pyrogallic-acid sodium-hydroxide as well as vacuum methods were also used. The cystine heart agar and one North's gelatin chocolate blood agar plate were incubated at 37.5°C. while another North's gelatin chocolate agar plate, one glucose plate, the leptospira medium, and the sodium-thioglycollate broth were incubated at room temperature (25 to 28°C.).

In addition to the culture work, slides of the triturated tick suspensions were made and stained with Ziehl-Neelsen's carbol fuchsin for the detection of acid-fast organisms.

Each strain of bacterium isolated was subjected to a complete cultural,

morphologic, physiologic and pathogenic examination. In determining physiologic properties the cocci were cultured in 12 differential media and all other forms were cultured in 30. For the pathogenicity tests each strain was injected into guinea pigs (intraperitoneally and subcutaneously), rabbits (intraperitoneally and intravenously), and mice (intraperitoneally) in varying dosages.

Results

Seventy-seven strains of bacteria were isolated and studied. These included 18 strains of gram-negative rods, 5 gram-positive rods, 47 gram-positive cocci, and 7 gram-positive spore-forming rods. No acid-fast bacteria were observed. Table 1 gives the number and percentage of bacteria in each morphologic group according to the sources of the ticks and to the condition of the ticks with respect to their blood meal.

When male and female unfed ticks were examined separately no essential difference was noted in the kinds of bacteria isolated. Most of the "fed" ticks tested were partially to fully engorged females.

In the case of those ticks which were dissected and the various organs or parts examined separately, cultivable bacteria were isolated from only the alimentary tract. It is known, however, that the bacterium causing tularemia may occur also in the Malpighian tubes and coelomic fluid of this tick (Francis, 1927).

Percentage of ticks harboring bacteria. In general, percentages of ticks harboring bacteria indicate that unfed ticks from vegetation contained the fewest bacteria (1.2 per cent) and the ticks from the domestic sheep harbored the most (17 per cent). If, however, one considers only the *engorged female* ticks from, for example, the domestic sheep, the percentage rises to almost 20. Since the latter ticks were examined in pairs, the percentage might have been higher if each tick had been examined individually.

The failure to recover bacteria from ticks from nature subsequently fed on domestic rabbits apparently does not present a true picture since well over one per cent of those reared on rabbits for use in production of Rocky Mountain spotted fever vaccine harbor bacteria.

Considering the data in general, it is convenient to suppose that each bacterial strain arose from one tick. Analyzed on this basis, the data give the following results: Of the 2016 unfed ticks, 1.6 per cent harbored bacteria, while of the 486 recently fed ticks, 9.1 per cent yielded bacteria. In other words, on the average, over 5 times as many fed ticks harbored bacteria as unfed ticks. There was no essential difference between laboratory-reared ticks and those collected in nature so far as the percentage of lots from which bacterial strains were recovered.

Kinds of bacteria. The 77 strains of bacteria were tentatively classified as to genus as follows: one of the genus *Achromobacter*, 4 *Alcaligenes*, 7 *Bacillus*, 2 *Corynebacterium*, 4 of the coliform group (probably *Escherichia*), 2 *Flavobacterium*, 47 *Micrococcus*, 4 *Pasteurella*, 2 *Pseudomonas*, and 2 gram-positive and 2 gram-negative rods were unidentified. The 4 strains of *Pasteurella* were all

TABLE 1

Number of bacterial strains, according to their morphologic group, isolated from adult D. andersoni obtained from various sources

BACTERIAL MORPHOLOGICAL GROUP	CONDITION OF TICKS WITH RESPECT TO BLOOD MEAL	LABORATORY REARED TICKS	TICK SOURCES: Ticks from vegetation: Unfed	Ticks from vegetation: Fed on G. P.'s	Ticks from vegetation: Fed on rabbits	Ticks from various animal hosts: Horse	Mule	Mt. sheep	Dom. sheep	Bear	Wild rabbits	TOTALS: According to whether ticks were fed or unfed	TOTALS: According to morphologic group	PER CENT
Gram-negative rods	Unfed	1	4			0	0	0	0	0	3	8	18	23.4
	Fed	0		1	0	2	0	0	6	0	1	10		
Gram-positive rods	Unfed	3	0			0	0	0	0	0	0	3	5	6.5
	Fed	0		0	0	0	0	0	2	0	0	2		
Gram-positive cocci	Unfed	14	5			0	0	0	0	0	0	19	47	61.0
	Fed	9		6	0	3	1	0	8	1	0	28		
Gram-positive spore-formers	Unfed	3	0					0	0	0	0	3	7	9.1
	Fed	2		0	0	0	0	1	1			4		
Acid fast rods	Unfed	0	0			0	0	0	0	0	0	0	0	0
	Fed	0		0	0	0	0	0	0	0	0	0		
Total number bacterial strains according to source	Unfed	21	9			0	0	0	0	0	3	33	77	100
	Fed	11		7	0	5	1	1	17	1	1	44		
Number of ticks examined from each source	Unfed	1065	777			57	2	52	12	26	25	2016	2502	
	Fed	20		157	94	109	6	6	88	4	2	486		
Total number of ticks		1085	777	157	94	166	8	58	100	30	27	2502		
Percentage of ticks harboring bacteria		2.9	1.2	4.5	0(?)	3.0	12.5	1.7	17.0	3.3	14.8			

P. tularensis isolated from the engorged female ticks obtained from domestic sheep. As far as the writer is aware, this is the first instance of *P. tularensis*

TABLE 2

Reactions of micrococci in differential media

GROUP		STRAINS PRODUCING ACID IN				STRAINS PRODUCING		NO. STRAINS TESTED
		Glucose	Lactose	Sucrose	Mannitol	H_2S	NO_2 from NO_3	
1. Pigment yellow to orange; gelatin not liquefied	Number	2	2	2	2	0	0	2
	Per cent	100	100	100	100	0	0	
2. Pigment yellow to orange; gelatin liquefied	Number	17	12	17	14	0	17	18
	Per cent	94.4	66.6	94.4	88.8	0	94.4	
3. Pigment red; gelatin not liquefied	Number	0	0	0	0	0	0	1
	Per cent	0	0	0	0	0	0	
4. No pigment, (white); gelatin not liquefied	Number	3	2	3	1	1	2	7
	Per cent	42.9	28.6	42.9	14.3	57.1	28.6	
5. No pigment, (white); gelatin liquefied	Number	18	13	18	12	5	16	19
	Per cent	94.7	68.4	94.7	63.1	26.3	90.0	
All Groups	Number	40	29	40	29	9	35	47
	Per cent	85.1	61.7	85.1	61.7	19.1	74.4	

TABLE 3

Bacterial strains, according to genus, tested for pathogenicity in guinea pigs, rabbits and mice

GENUS	NO. OF STRAINS TESTED	NO. OF STRAINS PATHOGENIC FOR		
		Guinea pigs	Rabbits	Mice
Achromobacter	1	0	0	1
Alcaligenes	4	0	0	3
Bacillus	7	2	1	3
Coliforms (probably *Escherichia*)	4	3	1	4
Corynebacterium	2	0	0	0
Flavobacterium	2	0	0	0
Micrococcus	47	0	4	4
Pasteurella	4	4	4	4
Pseudomonas	2	0	0	1
Unidentified	4	1	0	1
Totals	77	10	10	21

being isolated from *Dermacentor andersoni* by cultural methods. Parker and Spencer (1924) detected the tularemia organism in ticks by animal inoculation.

Excepting the four strains of *P. tularensis*, the only instances of multiple isolations were in the genus *Micrococcus*. Three species were each isolated twice, one 3 times, and one 5 times. Each isolation was from a different lot of ticks.

The largest number of bacteria of any morphologic group were of the genus *Micrococcus*. No attempt was made to classify them specifically, but following the general scheme of Bergey's Manual, they have been divided into 5 groups. Their characteristics are given in table 2. In the main they show the characteristics typical of saprophytic micrococci.

Pathogenicity tests. As shown in table 3, 10 of the 77 strains were pathogenic to guinea pigs, 10 to rabbits, and 21 to mice. In each case the criterion of pathogenicity was the death of the animal. With the exception of the 4 strains of *Pasteurella tularensis*, only two strains were pathogenic to all three animals: a coliform (probably *Escherichia*) and a *Bacillus* (*Bacillus megatherium*). In all, 23 different strains were pathogenic for at least one of the 3 species of test animals.

In view of the observations of many investigators that the fermentation of mannitol is a characteristic property of pathogenic staphylococci, it is interesting to note that while 29 of the cocci isolated produced acid in mannitol only 5 of these were pathogenic. The sixth pathogenic coccus did not ferment this carbohydrate.

Immunity tests. Because the rickettsia of Rocky Mountain spotted fever occurs spontaneously in *Dermacentor andersoni*, immunity tests against this disease were given to the guinea pigs which had been inoculated with the various strains of bacteria, in order to determine if there was any cross-immunity. To do this, 1.0 ml. of guinea pig blood virus of Rocky Mountain spotted fever (a *D. andersoni* strain) was inoculated into each pig which had survived the inoculation of bacteria for at least 20 days. In no case was any immunity shown.

OTHER MICROÖRGANISMS

Yeasts and molds. Examinations for these higher fungi yielded two strains of yeast but no molds. The two yeasts were not identified as to species but very probably belong to the tribe Saccharomyceteae. They were non-pathogenic for guinea pigs, rabbits, and mice.

Rickettsiae. The Rocky Mountain spotted fever rickettsia, *Dermacentroxenus rickettsii* Wolbach; the rickettsia of American Q fever, *Rickettsia diaporica* Cox; and a non-pathogenic rickettsia-like organism which has been studied by the writer and is the subject of a separate report, may occur spontaneously in *D. andersoni*. Several strains of Rocky Mountain spotted fever were isolated in guinea pigs inoculated with recently fed ticks from nature.

Because of the difficulty in differentiating microscropically between these three species in the tick, the presence or absence of rickettsiae was recorded for each lot of ticks examined without any regard as to the species of rickettisa concerned. Of the 145 lots examined, rickettsiae were observed in 94. The

number of Rocky Mountain spotted fever rickettsiae observed would, of course, be much smaller than this (see Parker and Spencer (1926)). All examinations for rickettsiae were made using both Macchiavello's and Giemsa's staining methods.

Spirochaetes. The suspension from each lot of ticks was stained with Giemsa's spirochaete stain. Of the 130 lots so examined, none showed spirochaetes.

Motile protozoa. The triturated tick suspensions from 130 lots of ticks were observed by the "wet mount" and occasionally by the "hanging drop" method for motile protozoa. In no case were any observed. These examinations were more or less cursory and should not be considered final. On several occasions forms which may have been encysted protozoa were seen but were not identified as such with certainty.

Bacteriophage. Though no routine examinations for bacteriophage were made, one strain was found associated with a strain of *Micrococcus* isolated from engorged female ticks obtained from domestic sheep. Apparently this is the first record of bacteriophage being isolated from ticks. About this same time, on two different occasions, a gram-negative short rod was isolated from another species of tick, *Dermacentor albipictus.* This bacterium was also heavily infected with a strain of bacteriophage.

ACQUISITION OF BACTERIA BY TICKS

During the course of the investigation, it became apparent that fed ticks harbored bacteria more frequently than did unfed ones. This fact suggested that ticks must acquire bacteria from their hosts while feeding. Since most of the strains isolated were saprophytes rather than pathogens, it did not seem reasonable that these bacteria were acquired with ingested blood. Therefore, the possibility that bacteria may be acquired adventitiously from the skin of the host was tested. Tests were made with *Serratia marcescens* and an unidentified *Micrococcus* isolated from *D. andersoni. Serratia marcescens* was chosen because of its pigment and because our studies had not yielded any organisms of this genus.

Cultures of these organisms were thinly smeared over the surface of the skin area on which the ticks were to feed. The test ticks were confined to this area by metal capsules and allowed to feed to engorgement. Their exteriors were then sterilized in the usual manner and their internal organs cultured on glucose beef-infusion agar. The three stages, larvae, nymphs, and adults were all treated in this manner. In every case it was shown that the test bacteria could be acquired from the skin surface of the host animal.

DISCUSSION

The literature contains several statements suggesting that the alimentary tract of ticks is invariably sterile. This general conclusion appears to be based on the work of Hindle and Duncan (1925) and Duncan (1926), who found the gut contents of *Argas persicus* to be devoid of bacteria and to contain a bacteriostatic principle. From the results of the present investigation, it is appar-

ent that in the case of *Dermacentor andersoni* the bacterial flora, consisting largely of organisms contained in the alimentary tract, is not an established one in the sense that it is in the case of certain of the Hexopoda. Instead, it appears that most of the bacteria this tick may harbor are the result of the fortuitous acquisition of these organisms from its host. This is shown by a comparison of the flora in *D. andersoni* and that found in some insects (Steinhaus, 1941). In the latter there were found 53 per cent gram-negative rods, 20.5 per cent gram-positive cocci, 12 per cent gram-positive short rods, and 14.5 per cent gram-positive spore-formers, while the flora of the tick consists of 23.4 per cent gram-negative rods, 61.0 per cent gram-positive cocci, 6.5 per cent gram-positive short rods, and 9.1 per cent gram-positive spore-formers.

Since it is generally accepted that the bacteria of the skin surfaces of most animals consists largely of cocci and since this appears to be the principal source of the *D. andersoni* flora, it is not surprising to find that over 60 per cent of the bacteria of this tick belongs to this group of microbes nor that most of them were non-pathogenic. Nevertheless, 23 different strains were pathogenic for one or more of the 3 kinds of test animals used.

Though we used media similar to that employed by Noguchi (1926) we were unable to recover, with certainty, the three organisms (*Bacillus rickettsiformis*, *Bacillus pseudoxerosis*, and *Bacillus equidistans*) isolated by him from *D. andersoni*. On several occasions we did isolate gram-negative short rods which were similar to those he described. Since apparently none of Noguchi's original cultures are available, it was not possible to make a close comparison.

SUMMARY

A study of the internal microbial flora of 2502 specimens of the Rocky Mountain wood tick, *Dermacentor andersoni*, obtained from various sources, yielded 77 strains of bacteria, 2 strains of yeasts, at least 1 rickettsia (that of Rocky Mountain spotted fever), 1 rickettsia-like organism, and 1 strain of bacteriophage. No acid-fast bacteria, molds, spirochaetes or motile protozoa were observed in any of the ticks examined. Each of the 77 strains of bacteria were studied in detail with respect to their cultural, morphologic, physiologic, and pathogenic characteristics.

Of 2016 unfed adult ticks, only 1.6 per cent harbored bacteria, but of 486 recently fed ticks, bacteria were found in a minimum percentage of 9.1. One possible explanation of the greater number of bacteria in recently fed ticks is the experimental finding that feeding ticks can ingest bacteria from surface of skin to which they are attached. The evidence suggests that the bacterial flora of *D. andersoni* is a fortuitous one consisting chiefly of adventitious organisms apparently acquired from its hosts.

REFERENCES

DUNCAN, J. T. 1926 On a bactericidal principle present in the alimentary canal of insects and arachnids. Parasitology, **18**: 238–252.

FRANCIS, E. 1927 Microscopic changes of tularemia in the tick *Dermacentor andersoni* and the bedbug *Cimex lectularius*. U. S. Pub. Health Repts., **42**: 2763–2772.

Hindle, E., and Duncan, J. T. 1925 The viability of bacteria in *Argas persicus*. Parasitology, **17**: 434–446.

Noguchi, H. 1926 Cultivation of rickettsia-like microorganisms from the Rocky Mountain spotted fever tick, *Dermacentor andersoni*. J. Exptl. Med., **43**: 515–532.

Parker, R. R., and Spencer, R. R. 1924 Tularaemia. XI. Tularaemia infection in ticks of the species *Dermacentor andersoni* Stiles in the Bitterroot Valley, Mont. U. S. Pub. Health Repts., **39**: 1057–1073.

Parker, R. R., and Spencer, R. R. 1926 A study of the relationship between the presence of rickettsia-like organisms in tick smears and the infectiveness of the same ticks. U. S. Pub. Health Repts., **41**: 461–469.

Steinhaus, E. A. 1941 A study of the bacteria associated with thirty species of insects. J. Bact., **42**: 757–790.

A NOTE ON ELASTICOTAXIS IN MYXOBACTERIA

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When myxobacteria of the family *Myxococcaceae* are grown on agar slants, a striking abnormality in the production of fruiting bodies is often apparent. The fruits formed along the line of inoculation are not scattered at random, but are definitely oriented in roughly parallel lines *at right angles* to the line of inoculation. Sometimes each line is composed of a series of the usual discrete, approximately spherical fruiting bodies, but in other cases the individual fruits become confluent, so that a ridge of microcysts is formed. Due to the spread of the vegetative swarm, fruits are also ultimately formed on the agar surfaces on either side of the line of inoculation; such fruits always exhibit the customary random distribution and normal shape. In fig. 1 is shown a slant culture of *Myxococcus fulvus* which illustrates this phenomenon. It was first observed in this species, but is also exhibited by all the other members of the *Myxococcaceae* which I have studied.

In seeking for an explanation of this behavior, it was at once apparent that chemical stimuli could not be invoked, since they would also have affected the fruiting process as it occurred in the regions of the slant surface away from the line of inoculation. The only conceivable difference between the streaked and unstreaked areas of the agar was a physical one. Accordingly, a series of experiments was performed in order to clarify the nature of the physical forces involved.

MATERIALS AND METHODS

As a test organism, *Chondrococcus* (*Myxococcus*) *exiguus* (Kofler) *nov. comb.*[2] was selected since it possessed several marked advantages for the purposes of the investigation. The fruiting bodies of this species are very small and inconspicuous, but they are formed in extreme abundance and in a comparatively short period of time on dung agar. The medium employed throughout was dung-decoction agar. The dung decoction was prepared by boiling horse or rabbit dung with twice the volume of water for 15 or 20 minutes, filtering, and diluting to an appropriate concentration (usually a tenfold dilution; this has to be varied somewhat, however, from one lot of dung to the next). To the diluted dung decoction 2% agar was added.

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[2] A complete description of this organism, together with the reasons for its separation from *C. coralloides*, with which it was combined by Jahn (1924) will be given in a later publication.

EXPERIMENTS

Experiment 1. Using sterile precautions, a rectangular piece of dung agar was cut out of a poured plate and placed over a glass rod in another petri dish as shown in fig. 2. The upper surface of the agar lying directly over the glass

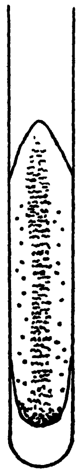


Fig. 1. Myxococcus fulvus, Showing the Distribution of Fruiting Bodies on an Agar Slant. Explanation in Text

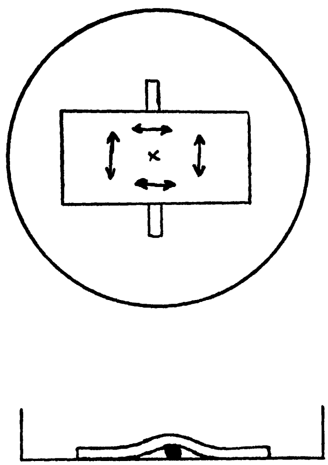


Fig. 2. The Set-up Used in Experiment 1, Viewed from Above and from the Side
The arrows in the upper diagram indicate the directions of stress on the agar surface.

rod was thus brought into a state of tension, which gradually decreased on either side and changed into a state of compression.

Under ordinary circumstances, a myxobacterial swarm will spread evenly in all directions from the point of inoculation, thus forming a circular colony. However, in this experiment the swarm behaved in an entirely different manner.

From the point of inoculation (marked X in fig. 2) it spread rapidly along the lines of tension but hardly at all across them; then, as it reached the region where the agar was in a state of compression, the direction of movement shifted by 90 degrees, so that the final shape assumed was that shown in fig. 3. Microscopic examination showed that the individual rods in the swarm were oriented parallel to the direction of movement and to the lines of force in any given region. Fruiting body formation occurred in irregular parallel lines which lay at right angles to the direction in which movement had taken place (fig. 3). The marked difference between fruiting body formation on stressed and unstressed media is shown in fig. 4. This experiment was repeated a number of times and always gave similar results. Occasionally the inoculation was made on the two ends of the agar strip which, lying flat on the floor of the petri dish, are subjected neither to tension nor to compression. In such cases, normal

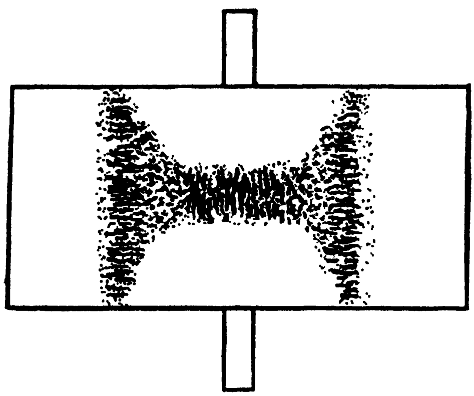


FIG 3 GROWTH OF C. EXIGUUS IN EXPERIMENT 1

The stippled area represents the swarm; the black lines within this area represent the fruiting bodies.

fruiting body formation occurred in the areas surrounding the inoculum, but changed sharply to the oriented pattern in those parts of the swarm which had reached and fruited on the areas of compression (fig. 5).

Experiment 2. Some sterile glass beads were scattered over the surface of a dung agar plate, which was then inoculated in the center. Normal random fruiting occurred in the regions free of beads, but around each bead the fruits were formed in irregular concentric rings, which gradually became less marked as the distance from the bead increased. The same phenomenon, although less sharply defined, is often observable in the immediate neighborhood of the inoculum, as can be seen in fig. 4.

Experiment 3. A dung-agar plate was inoculated in the center and incubated in a vertical position. Under these conditions no distortion of swarm or fruiting bodies took place, showing that the previously noted effects could not be attributed to gravitational forces.

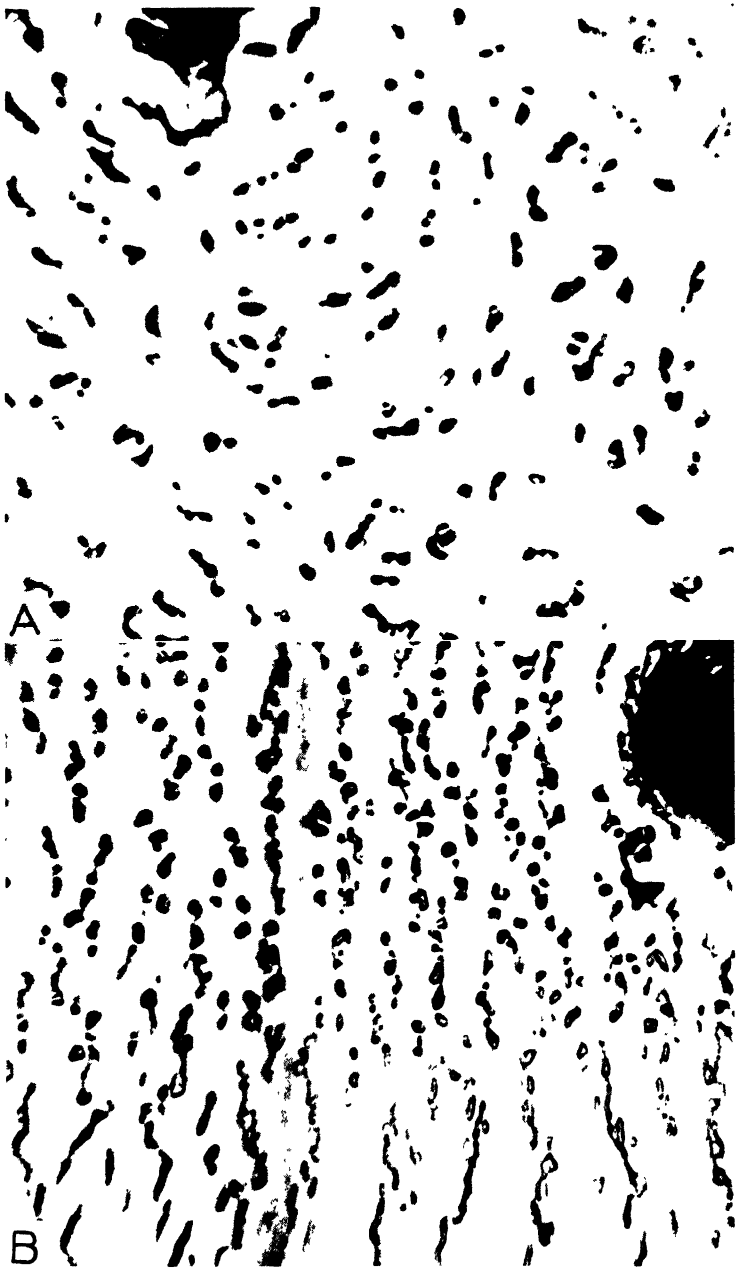


FIG. 4. FRUITING BODY FORMATION BY C. EXIGUUS

A On an unstressed surface. Note the indications of a concentric arrangement around the inoculum (large dark patch). B. On a surface subjected to tension.

INTERPRETATION AND DISCUSSION OF THE RESULTS

These experiments make it apparent that the peculiar manner of fruiting-body-formation first observed on slants is only a secondary effect, induced by oriented movement of the swarm. Furthermore, it is clear that the directive force which affects the vegetative cells is derived from the stresses in the agar gel which is acting as the substrate. Thus there are two distinct questions to be answered; what are the factors causing oriented movement of the vegetative cells? and how does the orientation of the vegetative cells alter the shape and arrangement of the fruiting structures?

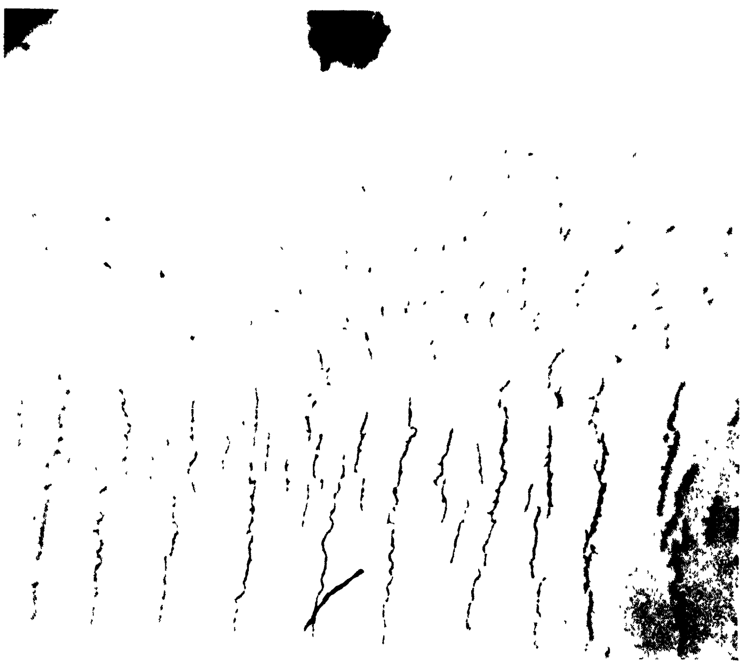


FIG. 5. FRUITING BODY FORMATION BY C. EXIGUUS, SHOWING THE CHANGE IN PATTERN AS THE SWARM SPREAD FROM AN UNSTRESSED SURFACE TO ONE SUBJECTED TO COMPRESSION

The orientation of *growth*, rather than *movement*, of living cells in response to stretching or compression of the substrate is a phenomenon which has long been known. In a masterly series of experiments, Jacobsen (1907) and Sergent (1906, 1907) showed that the growth pattern of *Kurthia* (*Zopfius*) *zopfii* in and on gelatin media is conditioned absolutely by the stresses which occur in the gelatin. In a number of ingenious ways they produced predictable stresses in the gelatin which were patterned in every detail by the growing *Kurthia* threads. Similar studies were later conducted by Kufferath (1911). More recently Weiss (1933, 1934, 1939) has studied extensively the oriented growth *in vitro* of vertebrate cells on coagulated plasma subjected to stresses. The patterns of development described by Weiss, Jacobsen, Sergent and Kufferath are broadly

comparable to those found in the present work, and there can be little doubt that in all these cases the factors responsible are the same.

Weiss has explained the directive effect of tensions as being due to the production of oriented structures in the substrate whose pattern is retraced by the developing cells; to this "ultrastructural organization" he has ascribed a major organizational rôle in embryological development. In order for this explanation to be valid, the substrate (ground substance) must be composed of large molecules possessing elasticity and capable of assuming a long chain structure under the influence of mechanical tension. These requirements are fulfilled by all three substrates on which the phenomenon has been observed. In coagulated plasma and gelatin the chemical skeleton consists of polypeptide chains, in agar of polysaccharide chains large polymeric molecules which can become oriented in the postulated manner. Particularly in the case of gelatin the potential orientability of the molecules has been clearly shown by X-ray analysis of stretched gels (for literature see Meyer and Mark (1930)). 2% agar gels under tension exhibit birefringence, although unstretched agar of the same concentration does not--an indication that here also the necessary oriented structures can be produced by mechanical forces.

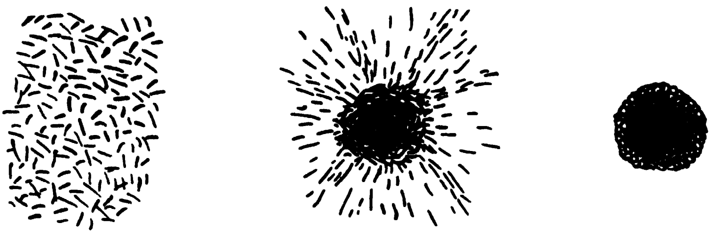
Perhaps the most convincing piece of evidence in support of Weiss' hypothesis is the observation, made by Weiss, Jacobsen and myself, that after the release of tension oriented growth or movement will still occur, due to the fact that the orientation in the substrate has become semipermanent. In the present work this is best shown by the preliminary observation of oriented fruiting along the line of inoculation of an agar slant long after the tension caused by pulling the loop over the agar has been removed. I have observed the same phenomenon on plates streaked with *Cytophaga krzemieniewskae* and *C. diffluens*. The swarm spreads far more rapidly along the streaks than at right angles to them, although the pattern eventually becomes obliterated by the decomposition of the agar. That syneresis cannot be a causative factor here is shown by the fact that oriented growth continues as long as the third day after inoculation.

Thus the primary effect of an oriented ultrastructure on the development of myxobacteria consists of an induced orientation in the swarm of vegetative cells. How does this in turn lead to the observed orientation of the fruiting bodies?

Under normal circumstances when fruiting occurs on an unstressed medium the vegetative cells in any given region of the swarm move towards one or more points on the surface of the agar and aggregate there to form the fruiting locus. The cells in these loci then gradually become transformed into microcysts. The vegetative rods in the area surrounding a fruiting locus orient and move towards it from all sides, just as if they were iron filings being attracted to the pole of a magnet. As a result, the mature fruiting body tends to be circular, or approximately so, in cross section. This is most perfectly exemplified in *Myxococcus fulvus* and *M. virescens; Chondrococcus exiguus* and the other *Chondrococcus* species which I have studied form somewhat irregular fruits. The normal fruiting process is shown diagrammatically in fig. 6.

On a substrate which is under tension the free movement of the vegetative cells in all directions necessary for the formation of round, discrete fruiting

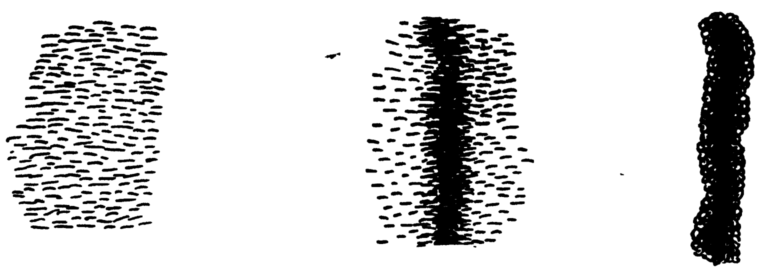
bodies is no longer possible or is greatly reduced; the cells can move back and forth parallel to the oriented ultrastructure of the substrate, but their movement across the lines of force is impeded. Thus when the stimulus to fruiting occurs, the vegetative cells are prevented from moving directly to the locus, and aggregate instead in irregular lines, as shown in fig. 7. If the orientation is weak, sufficient movement across the lines of stress may occur to make possible the formation of a row of discrete fruiting bodies, but on more strongly oriented substrates the final result will be a continuous ridge of microcysts.



A B C

FIG. 6. DIAGRAMMATIC REPRESENTATION OF FRUITING BODY FORMATION IN THE FAMILY MYXOCOCCACEAE AS IT OCCURS ON AN UNSTRESSED MEDIUM

A. Undifferentiated swarm. B. Vegetative cells moving to a fruiting locus. C. Mature fruiting body. A completely random arrangement of vegetative cells as shown in 4A is actually rarely seen, since there are usually waves and ridges of oriented moving cells all through the swarm; however, the cells in such regions are potentially capable of moving in any direction.



A B C

FIG. 7. DIAGRAMMATIC REPRESENTATION OF FRUITING BODY FORMATION IN THE MYXOCOCCACEAE AS IT OCCURS ON AN ORIENTED SURFACE

A. Undifferentiated swarm. B. Vegetative cells moving to a fruiting line. C. Mature fruiting body.

Jacobsen proposed the name *elasticotropism* for the directed growth of *Kurthia zopfii* through stretched gels; on the basis of his terminology, the directed movement of the myxobacterial swarm under similar conditions should be called an *elasticotaxis*.

SUMMARY

Swarm movement in myxobacteria belonging to the family *Myxococcaceae* is oriented parallel to stresses in the agar substrate, which results in a subsequent orientation of the fruiting bodies in irregular lines at right angles to the direction

of movement. The possible cause of this phenomenon, which may be termed an elasticotaxis, is discussed with particular reference to the hypothesis of Weiss.

REFERENCES

Jahn, E. 1924 Beiträge zur botanischen Protistologie. I. Die Polyangiden. Leipzig, Gebr. Borntraeger.

Jacobsen, H. C. 1907 Ueber einen richtenden Einfluss beim Wachstum gewisser Bakterien in Gelatine. Zentr. Bakt. Parasitenk., II, **17**, 53–64.

Kofler, L. 1913 Die Myxobakterien der Umgebung von Wien. Sitzber. Akad. Wiss. Wien, Math.-naturw. Klasse, Abt. I, **122**, 845–876.

Kufferath, H. 1911 Note sur les tropismes du *Bacterium zopfii* "Kurth." Ann. inst. Pasteur, **25**, 601–618.

Meyer, K. H., and Mark, H. 1930 Der Aufbau der hochpolymeren organischen Naturstoffe. Leipzig, Akademische Verlagsgesellschaft.

Sergent, E. 1906 Des tropismes du "Bacterium zopfii" Kurth. Première note. Ann. inst. Pasteur, **20**, 1005–1017.

Sergent, E. 1907 Des tropismes du "Bacterium zopfii" Kurth. Deuxième note. Ann. inst. Pasteur, **21**, 842–850.

Weiss, P. 1933 Functional adaptation and the rôle of ground substances in development. Am. Naturalist, **67**, 322–340.

Weiss, P. 1934 In vitro experiments on the factors determining the course of the outgrowing nerve fiber. J. Exptl. Zool., **68**, 393–448.

Weiss, P. 1939 Principles of development. New York, Holt.

CULTURE ON THE CHICK CHORIO-ALLANTOIS AS A TEST OF INACTIVATION OF VACCINIA VIRUS[1]

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There is considerable evidence of an empirical nature to indicate that the habitual use as a gargle and mouth wash, of an antiseptic solution so mild that it may be tolerated in contact with the mucous membrane, affords some degree of protection against upper respiratory infections. Thomson and Thomson (1932) writing in regard to prevention of colds have said "Probably one of the most important methods of reducing the chance of infection is by means of gargling the throat and treating the nasal passages with some mild antiseptic once or more times daily, more especially during epidemics. There has been a great deal of controversy over this procedure, but it seems to us extremely reasonable to suppose that just as we can prevent infection with venereal diseases through the application of antiseptics soon after exposure so we should be able to prevent droplet infection from respiratory diseases by gargling and washing out the nasal cavities with weak antiseptics." (Page 137.) This supposed favorable effect may be ascribed in part to the mechanical removal of microbes and their toxic products, in part to a favorable influence upon the blood supply to the mucous membranes and in part to a direct germicidal or restraining effect upon the micro-organisms themselves. It is possible that similar effects come into play when a gargle is employed by the physician to treat the early stages of a pharyngeal inflammation.

In immediate relation to the problem of preventing infections of the upper respiratory tract is the question whether such mild antiseptics may or may not exhibit an inactivating or restraining effect upon filterable pathogenic agents known as viruses, which are now generally regarded as important causative factors in some of these upper respiratory infections. Without experimental study, a mere survey of the known characters of these viruses suggested to us that an appreciable effect upon them by any such mild antiseptic was hardly to be expected. However, the experimental observations of others already recorded served to put us on guard against prophetic assumptions in regard to this matter.

In order to avoid, as far as might be, the theoretical entanglements and uncertainties which tend to confuse virus research, it seemed wise to initiate our studies by utilizing one of the best known of these agents giving rise to lesions in man, namely the virus of vaccinia. Our knowledge of this virus rests upon numerous repeated scientific observations by many workers over a con-

[1] Aided in part by the Virus Research Fund of the Lambert Pharmacal Company, St. Louis, Missouri.

siderable period of years in contrast to the somewhat insecure and unconfirmed doctrines which are current in respect to some other viruses. The individual virus particle of vaccinia, the Paschen body, may be accepted as a definite morphological entity. The lesions resulting from inoculation of the virus to man, calf, rabbit and to the membranes of developing chick embryos are well known. The latter lesions are distinctly visible and possess characteristics which permit them to be recognized with a fair degree of accuracy by the eye and sufficiently differentiated so that they can be recorded by photographs and preserved as permanent specimens in plastic mounts (Dunham, 1941). Vaccinia virus is apparently an altered type of the virus of variola and variola is a disease of man which may be acquired by inhalation of dry material harboring the virus or by the intentional application of such material to the mucous membrane of the upper respiratory region, as in the ancient practise of variolation. Another advantage is found in the harmless quality of this virus in relation to vaccinated human individuals, a matter of some importance in conserving laboratory personnel for work upon viruses. Furthermore the effect of antiseptics on vaccinia virus has already been studied to a considerable extent and further investigation of this matter cannot be expected greatly to alter the habits of the general population nor to revolutionize the practise of physicians. Hence the examination of this question may be undertaken in a quiet atmosphere of scientific enquiry sometimes referred to as academic.

Two strains of vaccinia virus have been used: (1) the glycerinated vaccine of the Department of Health of New York City, distributed for practical use in the Jennerian vaccination of the population and (2) an egg-adapted vaccinia virus, Strain CAEB, kindly supplied to us by Doctor Joseph E. Smadel of the Rockefeller Institute, New York City. This latter strain was received in the form of two specimens of virus-infected tissue. One was a chorio-allantoic membrane which had been preserved in the frozen state for more than a year. The other was a piece of similar tissue which had been dried rapidly while frozen and then preserved in the dry state. In our hands this virus has been propagated by serial inoculation onto the chorio-allantois of fertile eggs or by inoculation into the yolk of such eggs.

Culture technic. The technic of culture on the chorio-allantois is essentially that of Goodpasture, Woodruff and Buddingh (1932) as modified by Burnet (1936). However some new instruments have been devised and some technical modifications introduced (Dunham 1941, 1942.) An adequate supply of fertile eggs is obtained at regular intervals from chickeries which deal in eggs for hatching. Upon arrival at the laboratory each egg is numbered, recorded under the proper date and then placed in the egg incubator at a temperature of 100 to 101 degrees Fahrenheit (37.7 to 38.3 degrees C.). After development for 10 to 13 days, with daily turning, each egg is candled and the unsatisfactory sterile eggs or those with feeble or otherwise abnormal embryos are discarded. The side of the shell nearest the contained embryo is marked and this spot is held uppermost during the procedure of inoculation.

The uppermost surface of the shell is disinfected with 95 per cent alcohol and,

with aseptic precautions, is cut with a dental carborundum disc so as to separate a triangular segment of the shell measuring about 12 millimeters on each side. One is careful to avoid injuring the delicate underlying shell membrane. This operation is best done in or near an open funnel through which the dust is drawn away by adequate suction. The blunt end of the egg is then disinfected with alcohol and a minute opening is made with a small dental drill through the shell at this end so as to enter the normal air sac. The egg is then placed in our egg inoculator (fig. 1) so that the opening into the air space is covered by the suction disc which is connected to a continuous suction giving a negative pressure of about 50 millimeters of water. The suction line is provided with a safety by-pass to ensure that the negative pressure shall not become excessive. While the air space and, through it, the interior of the egg is subjected to this slight negative pressure, the loosened triangular piece of shell is lifted off with sterile forceps and placed in a petri dish. Then by means of our shell-membrane

FIG. 1. DUNHAM EGG INOCULATOR

teaser (fig. 2) a slit is produced by gently separating the diagonal fibers of the shell membrane; at either end of the slit a short incision at right angle to it is cut with sterile scissors and the membrane is torn by gentle traction so as to remove an approximately rectangular portion. At the initial perforation of the shell membrane air passes through and the immediately underlying vascular chorio-allantois falls away so that the risk of technical injury to this delicate and important structure is practically eliminated. The previously prepared inoculum is now dropped through the opening onto the chorio-allantois and the final droplet touched off by gentle contact with this membrane. The inoculum is conveniently handled in a tuberculin syringe with a 2 inch 20 gauge needle attached. After inoculation of the egg the triangular fragment of shell is replaced and sealed with liquid adhesive.[2] The hole at the blunt end is likewise sealed and the egg is returned to the incubator.

The subsequent period of incubation may be two or more days as desired. Ordinarily the inoculation of stock or control vaccine results in the formation of

[2] The liquid adhesive is obtainable from Johnson and Johnson Company, New Brunswick, N. J.

small opaque lesions on the chorio-allantois, which are about 0.5 millimeter in diameter at the end of two days and, after three days, 1 to 1.5 millimeters in diameter with visible necrotic centers. They tend to become confluent as they get older. For propagation of the stock vaccinia a period of three days seems best. For experimental tests of viability after possible inactivation somewhat longer incubation may be preferred. At the end of this period of incubation the membrane is harvested.

To harvest the membrane, one first cuts the shell with the carborundum disc, entirely around, just above the level of the chorio-allantoic membrane, and removes the upper portion of the shell so as to expose the inoculated area. By use of fine sterile scissors and forceps this portion of the chorio-allantois is removed and placed in sterile saline solution in a petri dish, where it is spread

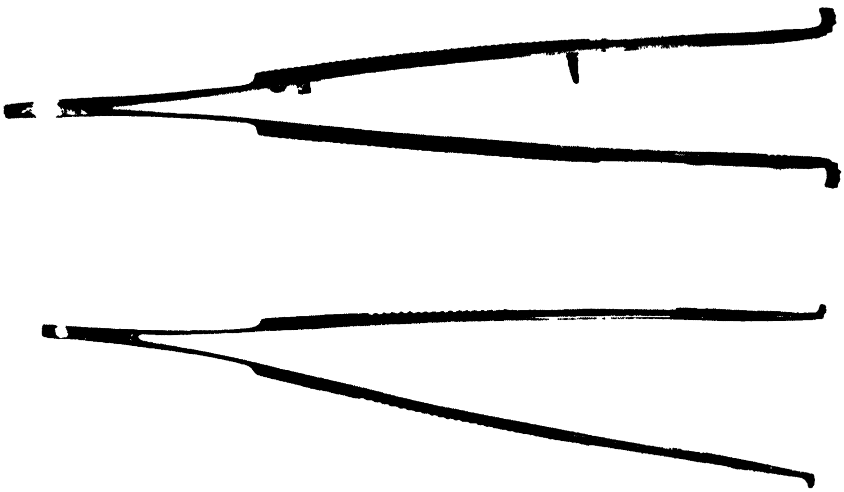

FIG. 2. SHELL-MEMBRANE TEASER, MADE BY BENDING THE TIPS OF STRAIGHT FORCEPS

out flat for study with a hand lens. Discrete lesions are counted and the approximate number of lesions in confluent areas estimated. The membranes are described for the record and sometimes are photographed in the salt solution or are dehydrated and embedded in plastic for permanent preservation. A diagrammatic drawing of the membrane is also made in the protocol as an aid to identification, thus permitting one dish to serve for holding several specimens. Sometimes the chorio-allantois is found thin and partly disintegrated by autolysis. Such eggs have evidently died shortly after inoculation, possibly because of operative trauma or some undetermined cause. They are discarded.

The virus suspension. For the preparation of a virus suspension to be used in testing antiseptics, several egg membranes with abundant vaccinia lesions are placed in a mortar and ground with sand. Tyrode solution, 2 milliliters for

each membrane used, is added to suspend the material and this suspension is then sedimented in the centrifuge at about 1,000 revolutions per minute for ten minutes. The supernatant, translucent but cloudy, light brown suspension is siphoned off and is used as the stock virus suspension. A freshly prepared suspension has been used for each experiment in the testing of the antiseptics.

Antiseptic agents. Several different chemical preparations were tested simultaneously against the same virus suspension and several eggs, usually six, were used to test the resulting possible inactivation of the virus by each antiseptic solution. The antiseptics were employed in their original state as supplied in the drug trade or were prepared in the laboratory from chemicals of high purity.

Technic of the tests. The antiseptic solution to be tested, 9 ml., was vigorously mixed with 1 ml. of the virus suspension and then, before sedimentation, a sample of this mixture was withdrawn into a tuberculin syringe fitted with a two-inch, gauge 20 needle. This syringe was inserted into a test tube which was immersed in ice water. The moment of mixing the virus with the antiseptic was recorded and the time of subsequent inoculation of this mixture into each egg was noted. The eggs were inoculated in rotation. Thus, in making a test of five antiseptic solutions and one control suspension the first egg received solution No. 1, the second solution No. 2 and so on, until each of the six had been inoculated into an egg. Then the seventh egg would be inoculated with solution No. 1. By this schedule solution No. 1 would be introduced into the first, seventh, thirteenth, nineteenth, twenty-fifth and thirty-first egg of the series. Thus, the time interval from the moment of mixing the antiseptic with the virus would be different for each egg but each of the solutions tested would be represented by an egg in each group of six eggs in the consecutive series.

The tests and their results. The most immediate practical question involved in these studies has concerned the possible virus-inactivating effect of Liquor antisepticus, (National Formulary, 1935) a solution which is extensively used in hospitals as a mouth wash and throat gargle. It is only fair to state that our experiments were undertaken with the expectation that the virus of vaccinia would not be perceptibly influenced by contact with this preparation. Hence a preliminary experiment was performed to demonstrate this assumed lack of inactivating power. A virus suspension was prepared from an egg membrane rich in lesions of vaccinia strain of the Health Department. Three solutions were tested (1) Tyrode solution as a control (2) Ethyl alcohol 25 per cent and (3) Liquor antisepticus. The eggs had been incubated eleven days; then inspected by candling and marked in the usual way. The mixture of Tyrode 9 ml. plus virus suspension 1 ml. after standing 21 minutes was inoculated to the chorio-allantois of egg 7627. In the same way egg 7608 was inoculated with the mixture of Alcohol 9 ml. plus virus suspension 1 ml. after this mixture had stood 26 minutes and egg 7620 was inoculated with the mixture of Liquor antisepticus, 9 ml. plus virus suspension 1 ml., after this mixture had stood 57 minutes. Additional eggs were inoculated at somewhat longer intervals as shown in table 1. The result, somewhat contrary to expectations, indicated a moderate degree of inactivation of vaccinia virus by the alcohol and complete

inactivation by the Liquor antisepticus in the exposure periods of 57 and 65 minutes.

Several repetitions of this experiment, exposing the virus for shorter time intervals, have consistently shown a greater degree of inactivation by the Liquor antisepticus as compared with the alcohol, although complete inactivation was not always attained. The protocol of one large experiment may serve as an example (table 2). In this experiment an attempt was made to test some of the individual constituents of Liquor antisepticus in concentrations of approximately the strength in the official solution. The Boric acid was dissolved in water. A mixture of Alcohol, 1 part, and Tyrode solution, 3 parts was employed to dissolve the Menthol, Thymol, Eucalyptol and Methyl salicylate. Along with these, Tyrode solution, Distilled water, Alcohol, 25 per cent in Tyrode solution, Alcohol 25 per cent in Distilled water and Liquor antisepticus were tested against the same virus suspension. The experiment required the inoculation of sixty eggs.

TABLE 1

Inoculations of vaccinia onto chorio-allantois. Preliminary test

Tyrode solution, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	21	34	49
	Lesions produced	400+	400+	400+
Alcohol 25 per cent, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	26	52	67
	Lesions produced	100+	25	60
Liquor antisepticus, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	57	65	
	Lesions produced	None	None	

To prepare the virus suspension, the membranes of three eggs with abundant vaccinia lesions, representing the virus of the Health Department, were used. Each final mixture was found free from ordinary bacteria by culture control. The inoculation dose for each egg was 0.025 ml. dropped onto the chorio-allantoic membrane. Results are shown in table 2.

In this experiment a long time was required for the technical procedure of inoculating the eggs. However, it is evident that the virus retained its potency in the Tyrode solution (391 minutes), in the Distilled water (392 minutes), in the Alcohol 25 per cent in Tyrode (390 minutes) and in the Alcohol 25 per cent in Distilled water (387 minutes). On the other hand, the virus was evidently inactivated by the Liquor antisepticus and apparently by the longer exposures to Boric acid. The Menthol, Thymol and Eucalyptol also seemed to have some inactivating effect, but the results were so irregular as to appear uncertain. For the Liquor antisepticus and the Boric acid it seemed that inactivation might be accomplished within a period shorter than any used in this experiment. Hence further tests were done at briefer exposure intervals for these two.

Six eggs were opened, ready for inoculation for each of these two antiseptic mixtures (Boric acid and Liquor antisepticus) and covered with sterile paper.

The virus suspension was then quickly mixed with the antiseptic. These eggs were inoculated after elapsed time of 1, 3, 5, 8, 10 and 12 minutes for the Boric

TABLE 2

Vaccinia in antiseptic mixtures inoculated onto chorio-allantois of fertile eggs, previously incubated 11 to 13 days

Tyrode solution, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	65	106	156	258	348	391	
	Lesions observed	16	Hem*	140+	97	130+	72	
Distilled water, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	63	101	157	253	343	386	392
	Lesions observed	20	100±	Dead	100±	120±	130±	150±
Alcohol 25 per cent in Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	64	105	158	258	347	390	
	Lesions observed	Dead	0	90	53	140±	120±	
Alcohol 25 per cent in Water, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	63	104	159	254	346	387	
	Lesions observed	40±	24	83	153±	Dead	130	
Liquor antisepticus, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	66	109	152	261	347	393	
	Lesions observed	0 (?)	0	Dead	0	0	0	
Boric acid in water, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	62	113	151	266	304	394	
	Lesions observed	3 (?)	Dead	Dead	Dead	0	0	
Menthol in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	66	110	151	263	347	394	
	Lesions observed	0 (?)	0	Dead	150+	45	0	
Thymol in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	66	111	149	265	303	396	
	Lesions observed	20	0	0	110±	Dead	15	
Eucalyptol in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	67	111	152	265	302	395	
	Lesions observed	15	117	Hem*	41	130	15	
Methyl salicylate in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	66	111	152	264	349	395	
	Lesions observed	1	10	Dead	Dead	150±	100±	

* Hemorrhage making it impossible to recognize lesions.

TABLE 3

Vaccinia in antiseptics inoculated to chorio-allantois after shorter intervals

Boric acid in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	1	3	5	8	10	12
	Lesions observed	250±	15	33	100±	0	23
Liquor antisepticus, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	2	3½	4	5	6	7
	Lesions observed	0	Dead	0	0	0	0
Alcohol, 25 per cent in Water, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	120	130	137	167	175	183
	Lesions observed	250±	250±	100±	7	24	15
Tyrode solution, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	124	134	141	171	18	189
	Lesions observed	300±	185±	50±	78	46	175±
Eucalyptol in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	130	138	146	176	185	192
	Lesions observed	154	246	270±	76	0	60
Thymol in Alcohol-Tyrode, 9 parts, plus Virus suspension, 1 part	Minutes after mixing	133	143	152	179	188	197
	Lesions observed	16	0	0	0	Dead	4 (?)

acid and after 2, 3½, 4, 5, 6 and 7 minutes for the Liquor antisepticus. Longer periods of exposure were allowed for the other antiseptics and for the control solutions. The time intervals and the results are shown in table 3. In this

experiment there was again a striking indication of rapid inactivation of the virus after it was mixed with the Liquor antisepticus.

The apparent inactivation of the virus by Liquor antisepticus was subjected to a further check. The chorio-allantoic membrane of an egg which had been inoculated with this mixture after an exposure period of 5 minutes, was preserved in Tyrode solution in the refrigerator for 48 hours, then ground with sand, suspended in 3 ml. Tyrode solution and inoculated onto the chorio-allantoic membranes of three more eggs. Two of these were opened after 48 hours and the third after four days. All three were alive and free from recognizable lesions of vaccinia.

TABLE 4

Inoculation of vaccinia virus CAEB onto chorio-allantois after exposure to diluted Liquor antisepticus, N.F. VII and to Distilled water

MIXTURE AND EXPOSURE	NUMBER OF LESIONS
Liquor antisepticus, N.F. VII, 90 per cent, 9 parts, plus Virus suspension, 1 part; after standing 30 seconds this was further diluted with Distilled water, 90 parts; then inoculated to four eggs	0 0 0 0
Liquor antisepticus, N. F. VII, 80 per cent, 9 parts, plus Virus suspension, 1 part; after standing 30 seconds this was further diluted with Distilled water, 90 parts; then inoculated to four eggs	0 0 0 0
Liquor antisepticus, N.F. VII, 10 per cent, 9 parts, plus Virus suspension, 1 part; after standing 20 minutes this was further diluted with Distilled water, 90 parts; then inoculated to four eggs	68 146 58 67
Distilled water, 9 parts, plus Virus suspension, 1 part; this was further diluted with Distilled water, 90 parts; then inoculated to four eggs	109 124 93 69

Repeated experiments of this type have shown some variations, particularly when much more dilute virus was used. In such a case the inactivation by Boric acid became evident after an exposure of 9 minutes and the Liquor antisepticus caused complete inactivation at 2 minutes while the virus remained active for two hours or more in the other solutions.

In the experiments so far, the briefest period of exposure of the virus to the antiseptic before inoculation into the egg was one minute. In order to test the possible effect of an even more brief exposure and at the same time to test the effect of dilution, a modification of the technic was introduced. Liquor antisepticus was prepared in the laboratory according to the officially revised formula (National Formulary, Interim Revision, 1939) effective July 1, 1940, a preparation to be designated as Liquor antisepticus, N.F. VII. Preliminary

tests showed that when this solution was diluted with nine parts of water it had no appreciable inactivating effect upon vaccinia virus. Hence it was possible to utilize this fact and to stop the action of the antiseptic on the virus at the end of any predetermined period of exposure, merely by diluting the test mixture. Subsequent inoculation onto the egg membranes was then carried out without undue haste. The results of a typical experiment of this kind, in which vaccinia virus CAEB was used, are shown in table 4. It is evident that 90 per cent and 80 per cent dilutions (actually reduced to 81 per cent and

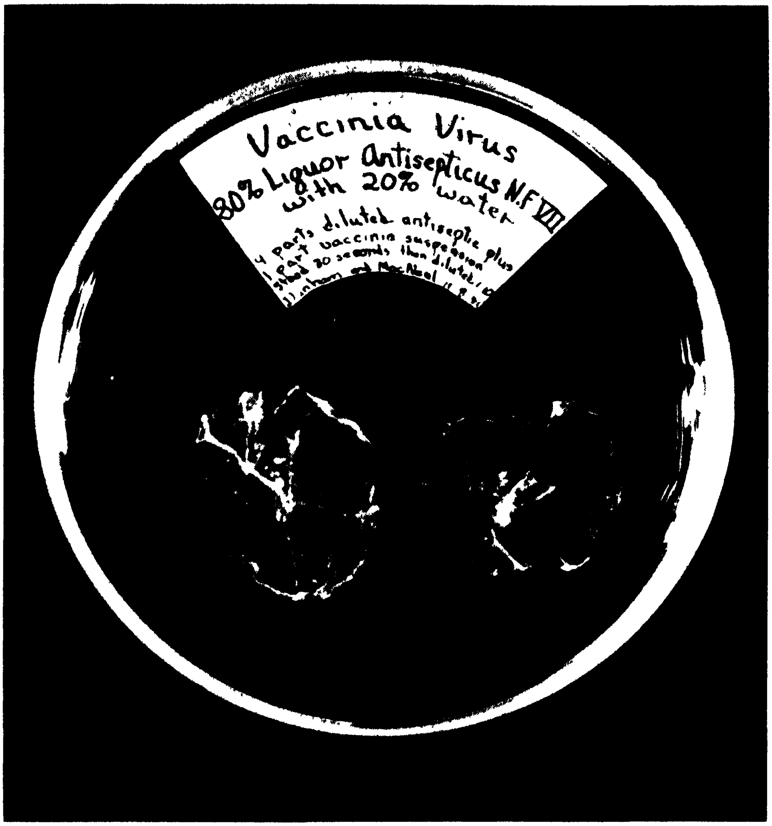

FIG. 3. EGG MEMBRANES FREE FROM LESIONS OF VACCINIA, TAKEN FROM TWO OF THE EGGS LISTED IN TABLE 4. LIQUOR ANTISEPTICUS, 80 PER CENT. MOUNTED IN PLASTIC.

72 per cent, respectively, by addition of the Virus suspension) of Liquor antisepticus, N.F. VII, inactivated the vaccinia virus in thirty seconds, at which time further action was halted by tenfold further dilution of these mixtures. The lack of potency after dilution is indicated by the behavior of the mixture in which 10 per cent Liquor antisepticus was tested against the virus for a period of 20 minutes, a much longer time than had been required to inoculate the eggs with the preceding test mixtures. Here the numbers of lesions developing on the inoculated egg membranes were of the same order as observed in the

control preparation using Distilled water. Photographs of membranes from this experiment, preserved in plastic, are shown in figures 3, 4 and 5.

Discussion. The criterion for inactivation of vaccinia virus employed has depended upon the production of vaccinia lesions upon the chorio-allantoic membrane of the chick embryos inoculated. Not too much should be claimed for the precision of the method. It may be assumed that the tissues of the chick have some resistance to the virus. Hence one is justified in concluding that inactivation of the virus for the chick embryo may not signify complete destruc-

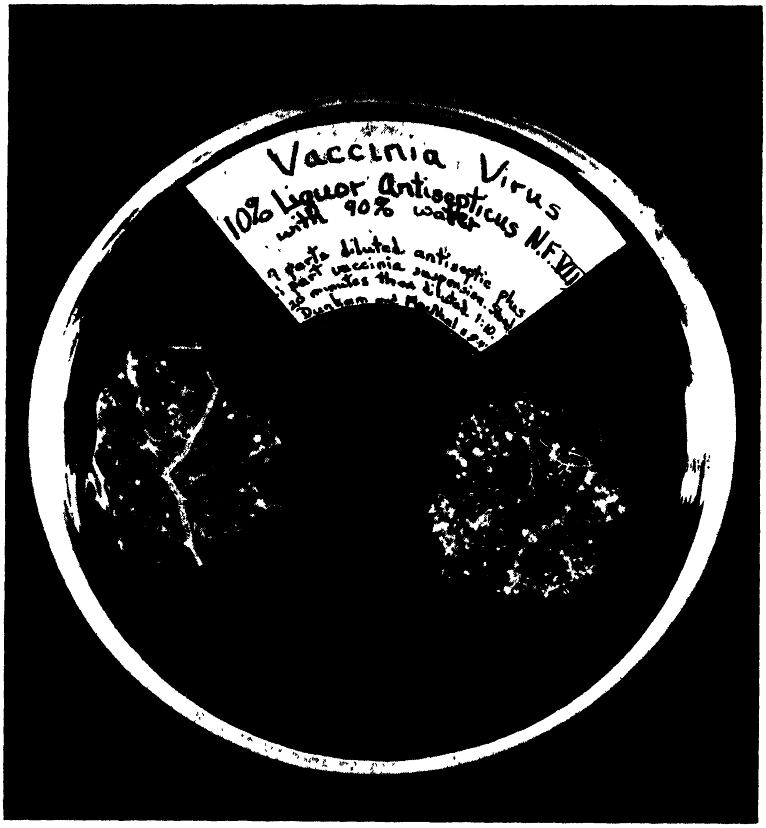

FIG. 4. EGG MEMBRANES WITH NUMEROUS LESIONS OF VACCINIA, TAKEN FROM TWO EGGS LISTED IN TABLE 4. LIQUOR ANTISEPTICUS, 10 PER CENT. MOUNTED IN PLASTIC.

tion of the virus. There is no reason to believe that the tissues of the chick embryo are more resistant in this respect than the tissues of other animals and it would seem fair to assume that the results have comparative value.

The experimental results were not entirely uniform. Irregularities in such work are, however, to be expected. We believe that they are in part due to the character of the virus preparation. This is a suspension of embryonic tissue elements containing the virus and not a suspension of separated virus particles. Hence it lacks uniformity of composition. To be sure, the gross bits of tissue

are thrown down by the centrifuge but it is by no means certain that small groups of tissue cells may not remain in the final suspension and thus offer a relative protection to virus particles in their interior. More exact and more regular results might be obtained by employing specially purified suspensions of the virus particles as used by Wilson Smith (1939) but such results would be of a less practical value because the natural dissemination of the virus takes place in association with tissue elements. For practical significance, therefore, the virus suspension as prepared would appear to possess some advantages over

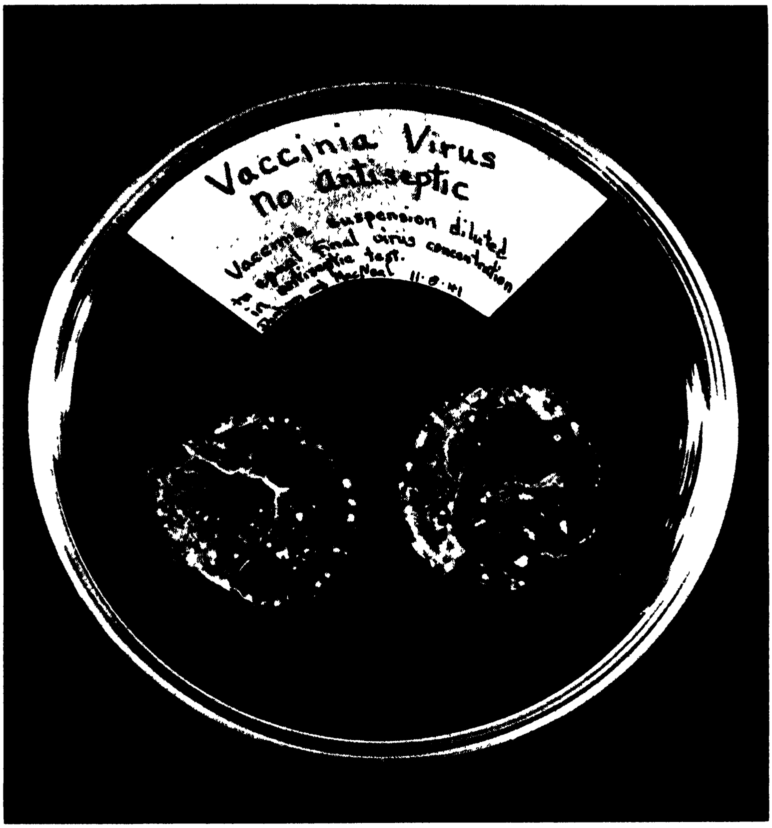

FIG. 5 EGG MEMBRANES WITH NUMEROUS LESIONS OF VACCINIA, TAKEN FROM TWO EGGS LISTED IN TABLE 4. DISTILLED WATER CONTROL MOUNTED IN PLASTIC.

a more uniform suspension of purified virus. Technical inequalities also introduce variations, especially the irregular distribution of the inoculum over the chorio-allantois, which has not been fully equalized.

The results show that vaccinia virus remains active for several hours at least when suspended in Tyrode solution, Distilled water, Alcohol 25 per cent in Tyrode solution, and in Alcohol 25 per cent in Water. On the other hand the virus is inactivated, as far as concerns its ability to produce lesions on the chorio-allantois of chick embryos, by Liquor antisepticus in less than a minute.

Some individual constituents of Liquor antisepticus, when employed separately, exhibit some tendency to inactivate the virus after longer periods of time. This seemed to be true particularly of the Boric acid, Thymol, Eucalyptol and Menthol. The action of each of these was however much less potent than the action of the Liquor antisepticus itself.

SUMMARY

1. The ability of Liquor antisepticus and of some of its constituents to inactivate vaccinia virus has been tested by inoculation onto the chorio-allantoic membranes of developing chick embryos.

2. When tested in this way it was found that the virus retains its activity very well when suspended in Tyrode solution, in Distilled water or in Alcohol 25 per cent. On the other hand it is quickly inactivated by Liquor antisepticus and appears to deteriorate less rapidly in solutions of Boric acid, Menthol, Thymol and Eucalyptol.

REFERENCES

BURNET, F. M. 1936 The use of the developing egg in virus research. Med Research Council (Brit), Special Rept Series No. 220, pp 11 15.

DUNHAM, WOLCOTT B. 1941a Permanent mounts of virus-infected chorioallantoic membranes. Science, **94**, 120

DUNHAM, WOLCOTT, B. 1941b Methods of virus culture. Proc. N. Y Soc Clin. Ophthalmol., Ser 3, **25**, 506.

DUNHAM, WOLCOTT B 1942 An egg inoculator for virus culture. J. Bact , **43**, 90.

GOODPASTURE, E. W , WOODRUFF, A M., AND BUDDINGH, A. J. 1932 Vaccinal infection of the chorio-allantoic membrane of the chick embryo. Am. J. Path. **8**, 271-281.

National Formulary. 1935 Sixth edition, Am. Pharm. Assoc., Official from June 1, 1936, pp. 219-220.

National Formulary. 1939 Interim revision announcement No. 2 Sixth edition J. Am. Pharm. Assoc , **28**, 1061-1062.

SMITH, WILSON. 1939 The action of bile salts on viruses. J. Path. Bact , **48**, 557 571.

THOMSON, DAVID, AND THOMSON, ROBERT. 1932 The Common Cold Annals of the Pickett-Thomson Research Laboratory, **8**, pp. XXIV + 738 Baillière, Tindall and Cox. London. (quotation on p. 137)

NITRATE, NITRITE AND INDOLE REACTIONS OF GAS GANGRENE ANAEROBES[1]

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In an attempt to work out a scheme for the rapid identification of the gas gangrene organisms (Reed and Orr, 1941), many of the published reports of nitrate reduction and indole formation by this group of organisms were found to be contradictory. The nature of the contradictions suggested that this was largely the result of faulty interpretation of results so clearly described for the nitrate reduction of bacteria in general by Conn (1936).

NITRATE REDUCTION

Woods (1938) found that washed suspensions of *Clostridium welchii* catalyzed the reduction of NO_3, NO_2 and NH_2OH to NH_3 by molecular hydrogen. Moreover, during the reduction of NO_3 to NH_3 he demonstrated the appearance and disappearance of NO_2. This made it probable that tests for NO_2 alone in growing cultures of this and related species would give little indication of the nitrate-reducing action of the organisms. A somewhat more detailed examination has therefore been made.

Cultures were tested for nitrite production in the following medium, Reed and Orr (1941):

Bacto tryptone	20 grams
Na_2HPO_4	2 grams
Glucose	1 gram
Agar	1 gram
KNO_3	1 gram
Water	1000 cc.

This was adjusted to pH 7.6 and autoclaved in deep tubes. In other experiments the medium without KNO_3 was tubed and autoclaved and filtered aqueous solutions of KNO_3 or KNO_2 added subsequently.

Qualitative tests for nitrites were made in the usual manner with Tittsler's (1930) sulphanilic acid, dimethyl-a-naphthylamine reagent. Where the qualitative nitrite reaction was negative, tests were made for nitrate by adding zinc dust, as suggested by Zo Bell (1932), to reduce remaining nitrate to nitrite. A quantitative nitrite reaction was devised by modifying the qualitative reaction. Aqueous solutions of KNO_2 were made up to contain 0.005 to 0.15 mg. per cent. To 5 ml. amounts, 1 ml. of Tittsler's mixed sulphanilic acid and dimethyl-a-naphthylamine reagent was added. After 15 minutes, readings were then made on a photoelectric colorimeter (Cenco photelometer with a No. 2 filter). The results

[1] Part of an investigation aided financially by the Canadian National Research Council.

were plotted on semi-log paper against the corresponding concentration of nitrite to form a basis for comparison with cultures. Cultures to be tested were centrifuged until clear and the supernatant diluted in water, usually 1–10, which largely obliterated the pale colour of the peptone solution. The test reagent was then added, readings made as in the case of the controls, concentrations read from the graph and multiplied by the dilution of the culture.

Twenty-one species belonging to the gas gangrene group of the genus *Clostridium* were grown in this medium and tested qualitatively for nitrites. Only five

TABLE 1

Qualitative determinations of nitrites and nitrates on cultures of twenty-one species of the genus Clostridium in a medium containing 100 mg. per cent KNO_3

	SPECIES	NO. OF STRAINS	TESTS FOR NO_2			TESTS FOR NO_3		
			1 day	2 days	5 days	1 day	2 days	5 days
Group I—NO_3 reduced rapidly, NO_2 present	*C. welchii*	8	+	+	+	Obscured by + NO_2 test		
	C. fallax	1	+	+	+			
	C. tertium	4	+	+	+			
	C. septicum	5	+	+	+			
	C. aerofoetidum	2	+	+	+			
Group II—NO_3 reduced rapidly, NO_2 absent	*C. sordelli*	4	–	–	–	Tr.	–	–
	C. bifermentans	2	–	–	–	–	–	–
	C. novyi	7	–	–	–	Tr.	–	–
	C. difficile	1	–	–	–	Tr.	Tr.	–
	C. paraputrificum	2	–	–	–	Tr.	–	–
	C. butyricum	4	–	–	–	Tr.	Tr.	–
	C. sporogenes	4	–	–	–	Tr.	–	–
	C. tetanomorphum	2	–	–	–	Tr.	–	–
	C. tetani	3	–	–	–	Tr.	–	–
	C. capitovalis	2	–	–	–	Tr.	–	–
Group III—NO_3 reduced slowly or not reduced, NO_2 absent	*C. carnis*	1	–	–	–	+	+	+
	C. histolyticum	4	–	–	–	+	+	+
	C. tyrosinogenes	2	–	–	–	+	+	+
	C. sphenoides	2	–	–	–	+	+	+
	C. cochlearium	5	–	–	–	+	+	+
	C. multifermentans	2	–	–	–	+	+	+

of the twenty-one species, *C. welchii*, *C. fallax*, *C. tertium*, *C. septicum* and *C. aerofoetidum*, as indicated in table 1, gave positive nitrite reactions. This is in agreement with most of the recorded findings, (Spray, 1936; Reed and Orr, 1941). But, as also indicated in table 1, ten of the twenty-one species, so break down the nitrate added to the medium that none is evident by the zinc-dust nitrite test. Cultures of the remaining six species (table 1), give positive tests for nitrate but show no accumulation of nitrites.

When quantitative tests for nitrite were made on cultures of the five species which gave positive nitrite tests (table 2) only small concentrations were found.

In the medium originally containing 100 mg. per cent of nitrate after 5 day's growth of *C. welchii* or *C. fallax*, less than 1 mg. per cent of nitrite was present and in cultures of *C. septicum* and *C. aerofoetidium* only about 10 mg. per cent of nitrite was present. This suggests that the NO_2 is itself being rapidly transformed and that the difference between this group (group I, table 1) in which NO_2 accumulated in measurable amounts and the next group (group II, table 1) in which no NO_2 could be detected is purely quantitative.

Quantitative nitrite tests of cultures of the twenty-one species after 1 to 5 days' growth in the peptone medium to which 10 mg. per cent of $NaNO_2$ was added, gave further evidence of the nature of the reaction (table 3). The almost complete disappearance of this amount of nitrite, probably by reduction to ammonia, from cultures of the five species which give positive qualitative nitrite tests when grown in a nitrate medium (group I, table 3) must indicate that ordinarily the reduction of nitrate to nitrite by these species is more rapid than the further reduction of nitrite. However, since the amount of nitrite

TABLE 2

Quantitative NO_2 in mg. per cent. Cultures in peptone medium containing 100 mg. per cent of KNO_3. Determinations were made at 1, 2 and 5 days after inoculation

SPECIES	MG. PER CENT OF NITRITE		
	1 day	2 days	5 days
C. welchii	0.20	0.51	0.58
C. fallax	1.20	0.63	0.66
C. tertium	3.10	3.20	4.10
C. septicum	10.80	11.70	12.35
C. aerofoetidum	9.85	10.10	12.80
Sterile medium	0	0	0

which accumulates is very small (table 2) it is quite likely that under different circumstances the rate of the two reactions may be equalized, in which case the qualitative tests for nitrite will become negative. This probably accounts for the considerable confusion which occurs in the literature.

Cultures of the ten species in group II, table 3 in media initially containing nitrate give *negative* qualitative reactions for both nitrates and nitrites. When grown in the peptone solution to which 10 mg. per cent of $NaNO_2$ is added the species of group II, like those of group I, show rapid breakdown of the nitrite. It therefore follows that when these species are grown in nitrate medium the rate of reduction of NO_3 to NO_2 must be equal to, or less than, the rate of reduction of NO_2. Here too is a likely source of error. It may be anticipated that under other circumstances the rates of the two reactions, relative to each other, may alter, in which case positive NO_2 reactions might be observed.

The species which make up the last group (group III, table 3) produce slow or negligible reduction of nitrite. Since in a nitrate-containing medium these species give negative qualitative reactions for NO_2 (table 1) and strongly positive

nitrate tests, it must be concluded that the reduction of NO_3 is also slow or does not occur.

INDOLE FORMATION

Hertzfeld and Klinger (1915) demonstrated a quantitative transformation of tryptophane to indole in cultures of *Escherichia coli*, due presumably to the enzyme tryptophanase recently described by Happold and Hoyle (1935). On

TABLE 3

Quantitative NO_2 in mg. per cent. Cultures in peptone medium containing 10 mg. per cent $NaNO_2$. Determinations made at 1, 2, and 5 day intervals after inoculation

	SPECIES	MG. PER CENT OF NITRITE		
		1 day	2 days	5 days
Group I—reduce NO_3 more rapidly than NO_2	*C. welchii*	Tr.	0	0
	C. fallax	0	0	0
	C. tertium	1.07	0.55	0.80
	C. septicum	0	0	0
	C. aerofoetidum	0	0	0
Group II—reduce NO_2 more rapidly than NO_3	*C. sordelli*	0	0	0
	C. bifermentans	0	0	0
	C. sporogenes	1.15	0.56	0
	C. novyi	0.42	0.23	0
	C. tetani	1.43	0	0
	C. tetanomorphum	0.05	0	0
	C. difficile	0	0	0
	C. paraputrificum	0	0	0
	C. butyricum	1.15	0	0
	C. capitovalis	5.30	1.50	0.05
Group III—reduce NO_3 and NO_2 slowly or not at all	*C. carnis*	6.90	4.30	3.80
	C. histolyticum	7.05	7.20	4.60
	C. tyrosinogenes	8.25	5.90	6.30
	C. sphenoides	8.70	7.50	7.00
	C. cochlearium	8.80	7.10	7.55
	C. multifermentans	9.45	8.10	7.70
	Sterile medium	9.40	8.30	7.80

the other hand, Happold and Hoyle (1936) have shown that *E. coli*, growing in a synthetic medium, slowly decomposes indole. It has also been shown by Sasaki (1923) that *B. subtilis*, and by Supniewski (1924) that *Pseudomonas aeruginosa* oxidizes indole to anthranilic acid, and by Gray (1928) that *Pseudomonas indoloxidans* will oxidize indole to indigotin. While it is unlikely that the anaerobic bacteria will oxidize indole, these observations do suggest a possible breakdown of indole as it is formed.

Indole determinations have been made on cultures of species of the genus

Clostridium after one to ten days' growth in the following medium, Reed and Orr (1941):

	grams
Bacto tryptone	20
Na_2HPO_4	5
Glucose	1
Agar	1
Sodium thioglycollate	1
Water	1000 ml.

The medium was adjusted to pH 7.6 and autoclaved in deep tubes. For some experiments this was modified by the addition of an aqueous solution of indole,

TABLE 4

Indole reactions of 17 species of Clostridia when grown for 1 to 10 days in a tryptophane medium without added indole and with 2 mg. and 10 mg. per cent of indole

	SPECIES	NO. OF STRAINS	MEDIUM CONTAINING NO ADDED INDOLE				MEDIUM CONTAINING 2 MG. PER CENT INDOLE				MEDIUM CONTAINING 10 MG. PER CENT INDOLE			
			1 day	2 days	5 days	10 days	1 day	2 days	5 days	10 days	1 day	2 days	5 days	10 days
Group I—indole accumulates	*C. sordellii*	3	+	+	+	++	++	++	++	+++	+++	+++	+++	++++
	C. bifermentans	2	+	+	+	±	++	++	+	+	+++	+++	++	+
	C. capitovalis	2	+	+	+	++	++	++	++	+++	+++	+++	+++	++++
	C. sphenoides	2	+	+	+	+	++	++	++	++	+++	+++	+++	+++
Group II—indole used as rapidly as formed	*C. welchii*	3	0	0	0	0	+	0	0	0	+++	++	+	+
	C. sporogenes	2	0	0	0	0	0	0	0	0	0	0	0	0
	C. novyi	2	0	0	0	0	0	0	0	0	++	+	+	0
	C. tetani	2	0	0	0	0	0	0	0	0	+	0	0	0
	C. carnis	1	0	0	0	0	+	0	0	0	++	++	0	0
	C. fallax	1	0	0	0	0	+	0	0	0	++	++	+	0
	C. septicum	2	0	0	0	0	0	0	0	0	++	++	+	+
	C. multifermentans	2	0	0	0	0	+	0	0	0	++	++	+	+
	C. histolyticum	2	0	0	0	0	0	0	0	0	++	++	+	+
	C. tetanomorphum	2	0	0	0	0	+	0	0	0	++	++	+	0
	C. difficile	1	0	0	0	0	+	0	0	0	+	0	0	0
	C. aerofoetidum	2	0	0	0	0	+	0	0	0	++	++	+	0
	C. paraputrificum	2	0	0	0	0	+	0	0	0	++	++	+	0
	Sterile medium		0	0	0	0	++	++	++	++	+++	+++	+++	++

sterilized by filtration, to provide 2 mg. per cent or 10 mg. per cent of added indole.

Qualitative tests for indole were made on cultures after 1, 2, 5 and 10 days' incubation with Fellers and Clough's (1925) modification of Ehrlich's reagent.

A summary of the results of these tests on cultures of several strains of seventeen species grown in the tryptophane medium without added indole, with 2 mg. per cent, and 10 mg. per cent of indole are shown in table 4. From the first column it is apparent that indole accumulates in measurable amounts in cultures in the tryptophane medium of only four of the seventeen species tested, *C. sordellii*, *C. bifermentans*, *C. capitovalis*, and *C. sphenoides*. This is in agreement with previous reports (Spray, 1936; Reed and Orr, 1941).

The remaining thirteen species tested, it will be observed from the table, do not accumulate measurable amounts of indole in the tryptophane medium. But, it is also apparent from the table, that indole added to the medium in which these organisms are grown rapidly disappears. There is a great difference, however, in the rate of disappearance. In the medium with 2 mg. per cent of added indole, five species bring about its disappearance in twenty-four hours and all thirteen species in two days. In the media with 10 mg. per cent of indole, *C. sporogenes* causes complete disappearance in twenty-four hours, but in cultures of four species, detectable amounts are still present after ten days' incubation.

It seems probable therefore that all thirty-three cultures belonging to the seventeen species tested produce indole from tryptophane, but that all except four species either utilize or break down indole as rapidly as formed.

It is apparent from the qualitative reactions indicated in the table that a species like *C. bifermentans* breaks down or utilizes indole but that ordinarily the rate of indole formation from tryptophane is more rapid than the rate of disappearance. Under other conditions it is possible that the rates of the two reactions may be equalized. Conversely *C. welchii* brings about the disappearance of indole more slowly than most of the other species grouped with it, yet rapidly enough to prevent the accumulation of indole from tryptophane. A slight modification in the rate of either of these reactions would probably result in indole accumulation.

CONCLUSIONS

It is shown that the gas gangrene species of the genus *Clostridium* fall into three groups in respect to the reduction of nitrates and nitrites: (1) Five species which reduce both NO_3 and NO_2 but in which the rate of reduction of NO_3 is ordinarily more rapid than the reduction of NO_2; as a result qualitative reactions for NO_2 are generally positive. (2) Ten species which also reduce both NO_3 and NO_2 but in which the rate of reduction of NO_2 is equal to or greater than the rate of NO_3 reduction. As a result qualitative tests for NO_2 are ordinarily negative. (3) Six species which fail to reduce both NO_3 and NO_2 or reduce them at an equally slow rate, so that qualitative tests for NO_2 are regularly negative.

It is also shown that these species fall into two groups in respect to indole formation: all species tested break down or utilize indole but (1) four species grown in a tryptophane medium give positive indole tests in which the rate of indole formation is greater than the rate of indole breakdown (2) thirteen species in which the rate of indole formation is equal to or less than the rate of indole breakdown.

REFERENCES

Conn, H. J. 1936 On the detection of nitrate reduction. J. Bact., **31**, 225–233.

Fellers, C. R., and Clough, R. W. 1925 Indole and skatole determinations on bacterial cultures. J. Bact., **10**, 105–133.

Gray, P. H. H. 1928 The formation of indigotin from indole by soil bacteria. Proc. Roy. Soc., London, **102**, 263.

HAPPOLD, F. C., AND HOYLE, L. 1935 The coli-tryptophane-indole reaction: I. Enzyme preparations and their action on tryptophane and some indole derivatives. Biochem. J., **29,** 1918–1926.

HAPPOLD, F. C., AND HOYLE, L. 1936 The coli-tryptophane-indole reaction: II. The non-production of tryptophanase in media containing glucose. Brit. J. Exptl. Path., **17,** 136–143.

HERTZFELD, E., AND KLINGER, R. 1915 Quantitative Untersuchungen uber den Indol- und Tryptophanumsatz der Bacterien. Zentr. Bakt., Parasitenk., I, orig., **76,** 1.

REED, G. B., AND ORR, J. H. 1941 Rapid identification of gas gangrene anaerobes. War Medicine, **1,** 493–510.

SASAKI, T. 1923 Über die Bildung des Anthranilsaure aus *l*-tryptophan durch Subtilisbacterien. J. Biochem., **2,** 251.

SPRAY, R. S. 1936 Semisolid media for the cultivation and identification of the sp rulating anaerobes. J. Bact., **32,** 135–155.

SUPNIEWSKI, J. 1924 Der Stoffwechsel der Zyklischen Verbindungen bei *B. pyocyaneus.* Biochem. Z., **146,** 522.

TITTSLER, R. P. 1930 The reduction of nitrates to nitrites by *Salmonella pullorum* and *Salmonella gallinarum.* J. Bact., **19,** 261–267.

WOODS, D. D. 1938 The reduction of nitrate to ammonia by *Clostridium welchii.* Biochem. J., **32,** 2000–2012.

ZO BELL, C. E. 1932 Factors influencing the reduction of nitrate and nitrite by bacteria in semi-solid media. J. Bact., **24,** 273–281.

THE ERWINIA-COLIFORM RELATIONSHIP

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Erwinia, a genus of bacterial plant pathogens, has long been recognized as closely related to the *Escherichia-Aerobacter* group. This view, although readily accepted, has been the object of little comparative study; the relationship is usually taken for granted, the result being a relatively poor understanding of the similarities of the two groups. On the basis of its disease-producing ability in plants, Bergey *et al.* (1939) gives the group tribal ranking, Erwineae, in the family Enterobacteriaceae. Other than the obvious facts that the species of *Erwinia* are peritrichously flagellated, gram negative, do not form spores and ferment many carbohydrates, including lactose, our knowledge of the relationship is meager. The possibility that the *Erwinia*[1] might be coliform organisms has been given comparatively little thought.

Stuart *et al.* (1938, 1940) recognized the possibility of *Erwinia* cultures being confused with coliform organisms, or vice versa, and included some plant cultures in their studies. Stanley (1939) likewise declared the soft-rot group to belong to the colon-typhoid-dysentery group, and suggested that organisms identified as *Erwinia* might be *Escherichia coli*, the latter interpreted in a broad sense. According to Parr (1939), F. D. Chester stated that the genus *Erwinia* was established on a purely utilitarian basis and had no genetic standing.

Dowson (1939), following the usual British practice, advocated the inclusion of the soft-rot pathogens in the genus *Bacterium* Lehmann and Neumann. By this procedure he recognized the apparent close relationship of the coliform and *Erwinia* groups, as the former are likewise placed in this genus.

Unfortunately, the biochemical characteristics of the soft-rot organisms are as variable as those of coliform isolates (Stanley 1939, Elrod 1941). It has been shown, especially by Stanley, that fermentative shifts are common, tending to throw a culture from one species to another. These changes usually concern a change from an aerogenic to an anaerogenic state, although some variation has been noted in the methyl-red, Voges-Proskauer and citrate tests. Elrod (1941) found that the soft-rot group is as antigenically heterogeneous as the coliform group.

In the light of our abundant knowledge of the coliform group, it was decided first to employ "coliform" methods in an attempt to arrive at a better understanding of the *Erwinia-Escherichia-Aerobacter* relationship. The recent work of Stuart, Mickle and Borman (1940) on aberrant coliforms offers a way of

[1] The term *Erwinia* in this paper is taken to mean the so-called soft-rot group. This group includes: *Erwinia carotovora* (Jones) Holland, *Erwinia aroideae* (Townsend) Holland, *Erwinia solanisapra* (Harrison) Holland and *Erwinia phytophthora* (Appel) Bergey *et al.*

grouping the soft-rot organisms with the Eschericheae. Accordingly, the following tests were performed: 1) lactose fermentation, 2) IMViC (indole, methyl-red, Voges-Proskauer, citrate) plus cellobiose fermentation, and 3) gelatin liquefaction. Our knowledge of the reactions of coliform cultures in these tests was abundant and there was no need to repeat this work.

Moreover, the main criterion of the genus *Erwinia*, i.e., the ability to attack plant tissues, was applied to coliforms. This characteristic is usually associated with the ability of the organisms to breakdown pectin (Chester in Bergey *et al.*, 1939). Why this idea is prevalent is difficult to fathom, unless it arises from a lack of understanding of pectic compounds in the plant. It is known, although not widely recognized, that the enzymes protopectinase (which brings about tissue disintegration) and pectinase (which is associated with the breakdown of pectin or pectic acid) are different entities (Davison and Willaman, 1927). Nevertheless, it was thought advisable to determine whether or not any correlation did exist between the ability to disintegrate vegetable tissue and the fermentation of pectin. If such were the case, we would have a readily accessible method for distinguishing between *Erwinia* and coliform cultures.

Cultures used. The soft-rot isolates used in this study have been tested for rotting ability many times and until January, 1941, all exhibited this property. Since then, three (CA1, EC1, and CA2) of the cultures have become avirulent. Nevertheless, these organisms have been incorporated in order to compare virulent with avirulent forms. The cultures used were, with a few exceptions, identical with those studied by Elrod (1941) in a biochemical and serological survey of the soft-rot group. They were all actively motile.

EXPERIMENTATION

All of the biochemical and rotting tests were conducted in duplicate at both 20°C and 37°C to ascertain if *Erwinia* exhibited the same temperature differences noted by Stuart *et al.* (1940) in their study of aberrant coliforms.

a. Lactose fermentation

As is seen in table 1, all of the *Erwinia* cultures were able to ferment lactose at 20°C. The time necessary for the production of acid (Durham fermentation tubes with brom-cresol-purple as an indicator were employed) varied considerably. Several cultures, NZ, 4, 5, 11, EC1 and B81, formed acid in 24 hours. The slowest fermenting culture was ES which required seven days to produce a definite acid reaction. A small bubble of gas was produced by the tenth day. Gas was formed by seven of the strains but never in a quantity exceeding 20 per cent of the vial. This gas formation was slow and only an immeasurable amount was formed in 48 hours. Whereas all the cultures fermented the disaccharide at 20°C, five failed to do so at 37°C.

b. IMViC plus cellobiose

None of the cultures tested for the formation of indole with Kovac's reagent formed this substance in five days' time in tryptophane broth (table 1). This

result is in keeping with most of our information concerning indole production by soft-rot organisms. Exceptions can be found in the literature, most notably in Bergey *et al.* (1939) where *Erwinia solanisapra* and to some extent *E. carotovora* are said to form small amounts of indole. Stanley, however, with over 100 cultures found but two organisms which formed indole and one of these was a non-lactose-fermenter. Bonde (1939) was able to demonstrate indole production by several soft-rot strains by means of Kovac's test, although several other tests had proved negative.

TABLE 1

Lactose, IMViC and cellobiose reactions by Erwinia isolates at 20°C and 37°C

ORGANISM TESTED	LACTOSE		INDOLE		METHYL RED		VOGES-PROSKAUER		CITRATE		CELLOBIOSE		IMViC + CELLOBIOSE AT TEMPERATURE OF GREATEST ACTIVITY
	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°	
CA1	AG	AG	−	−	±	+	+	+	+	+	AG	AG	−++++
NZ	A^b	A	−	−	+	+	−	−	+	−	A	A	−+−+A
496	A	−	−	−	+	−	−	−	−	−	A^b	A	−+−−+
494	A	−	−	−	+	−	−	−	+	−	A	−	−+−+A
WV3	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
EC1	AG	AG	−	−	−	−	+	+	+	+	A^b	+	−−+++
EC2	A^b	A	−	−	+	−	−	−	+	−	A	A	−+−++
EC4	A	A	−	−	−	−	−	±	+	−	A	A	−−++A
EC3	A	A	−	−	−	−	−	±	+	−	A	A	−−++A
B81	A	A	−	−	+	±	+	±	+	−	A	A	−+++A
5	A	A	−	−	+	−	−	−	+	−	A	A	−+−+A
11	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
EA	A	A	−	−	+	−	+	−	+	−	A	A	−+++A
ES	A^b	−	−	−	+	−	−	−	−	−	A	−	−+−−A
495	A	−	−	−	+	±	−	−	+	−	A^b	A	−+−++
EC	A	A	−	−	+	+	−	−	+	+	A	A	−+−+A
WV6	A	AG	−	−	−	−	+	+	+	+	AG	AG	−−+++
CA2	AG	−	−	−	+	±	+	+	+	+	AG	−	−++++
4	A	A	−	−	+	−	−	+	+	−	A	A	−+−+A

AG = acid and measurable gas. − = no acid or gas or no reaction.
A^b = acid and bubble of gas ± = weak reaction.
A = acid only. + = strong reaction.

The medium used in both the methyl-red and Voges-Proskauer test was Difco's M.R.-V.P. medium. Methyl-red tests were made at 48 and 96 hours, while acetylmethylcarbinol was tested for each day for one week. In the latter, Barrit's α-naphthol test was used. The Voges-Proskauer test was fairly constant at the two temperatures employed, while a considerable difference was noted in the methyl-red reaction between 20°C and 37°C. A stronger acid reaction with ten of the cultures was produced at the lower temperature. In the final analysis, eleven cultures were MR+, VP−; four, MR−, VP+; and four, MR+, VP+ (table 1).

The methyl-red and Voges-Proskauer tests seldom have been used in regard to *Erwinia* isolates. Dowson (1939) lists *E. aroideae* as VP+, MR− and

E. carotovora, VP−, MR+. Whether more than one culture of each was used in drawing this conclusion is not known. Stanley found his cultures to be extremely variable in the methyl-red test when tested over a period of years. Many of his cultures were VP−, MR+, many VP+, MR−, and a larger number VP−, MR−, while only a few were VP+, MR+.

All of my cultures but two (ES and 496) were positive on Simmon's citrate agar at 20°C. At 37°C, however, only seven of the strains were able to utilize the citrate as the sole carbon source (table 1). The majority of Stanley's cultures were citrate-positive at 20°C.

TABLE 2

Gelatin liquefying, pectin fermentation and rotting tests of Erwinia and coliform cultures

ORGANISM TESTED	GELATIN	PECTIN	CARROT	TURNIP
CA1	+	+	−	−
NZ	+	+	+	+
496	+	−	+	+
494	+	+	+	+
WV3	−	+	+	+
EC1	+	−	−	−
EC2	+	+	+	+
EC3	+	+	+	+
EC4	+	+	+	+
B81	+	+	+	+
5	+	+	+	+
11	+	+	+	+
EA	+	+	+	+
ES	+	+	+	+
495	−	+	−	+
EC	+	−	+	+
WV6	−	−	−	+
CAZ	+	+	−	−
4	+	+	+	+
50 representative coliforms		22*	0*	0*

* Number of cultures reacting positively.

As in the case of lactose, all of the strains fermented cellobiose at 20°C. In some cases the reaction proceeded rapidly; in others, very slowly. Three isolates failed to ferment the sugar at 37°C (table 1).

c. Gelatin liquefaction

One of the differences between *Aerobacter aerogenes* and *A. cloacae* is the ability of the latter to liquefy the gelatin medium. This reaction, however, is not a constant characteristic, there being found numerous non-liquefying strains of *A. cloacae*. Gelatinolysis with this organism usually proceeds slowly, although there are exceptions.

In gelatin stab cultures, sixteen of the nineteen isolates were able to hydrolyze the medium (table 2). For the most part, this liquefaction was rapid,

being complete in 2–3 days at 20°C. There were a few cultures that required 10–14 days before hydrolysis was detectable. Frazier gelatin-agar plates also proved the remaining five strains to be negative. Bonde (1939) has indicated that all of his isolates liquefied gelatin, and, this is usually thought to be true of soft-rot organisms.

d. Pectin fermentation

Inasmuch as the reducing sugars associated with pectin are soluble in 80 per cent alcohol, several soakings and washings with this solvent eventually removed any of these contaminating substances. The usual procedure was to place a weighed sample of the granulated pectin in a flask to which considerable 80 per cent alcohol was added. The mixture was shaken thoroughly and then incubated 12 hours with occasional shaking. The alcohol was filtered off, fresh alcohol added and the flask again incubated 6–8 hours. The mixture was filtered and the pectin on the filter paper washed 6–7 times with 80 per cent alcohol and twice with 95 per cent. The alcohol-moist pectin was then placed in a sterile petri dish and heated to dryness at 37°C. A 5 ml. sample of the last 80 per cent alcohol filtrate was evaporated to dryness and a qualitative Benedict's test performed with the residuum; this invariably proved negative for reducing sugars. This procedure shortens the time of McFadden's method (1941) five or six days.

A basic synthetic medium was made up of 0.2 g. magnesium sulfate, 0.1 g. calcium chloride, 0.2 g. sodium chloride and 0.2 g. dipotassium phosphate per liter. This was sterilized by filtration through a Berkefeld N filter. The pectin was added so that the final concentration was approximately 0.5 per cent. Brom-cresol-purple was used as an indicator. On the addition of pectin, the medium became acid and it was necessary to adjust to neutral with sterile NaOH. After tubing aseptically, the tubes were incubated 48 hours at 37°C and 3–4 days at 20°C. The percentage of contaminated tubes was about 5 per cent; fairly effective sterilization of the pectin had taken place during the purifying process.

Four tubes of this medium were inoculated with each *Erwinia* and coliform culture, two incubated at 37°C and two at 20°C for one week. All but two (EC and 496) of the actively rotting organisms fermented pectin. On the other hand, there were a great number of *Aerobacter* and intermediate coliform cultures which fermented the carbohydrate. Only three strains of *Escherichia coli* were positive in the pectin medium; all of these were of fecal origin. Among the fermenting *Aerobacter* cultures a large number were derived from feces. It is apparent that there is no correlation between the ability to ferment pectin and the rotting of carrot or turnip (table 2).

e. Pathogenic action on carrot and turnip

As a means of testing the pathogenicity of the organisms, both carrots and turnips have been used. Jones' (1905) method was used to test the pathogenicity of the organisms against carrot and turnip. The vegetable tissue was sectioned aseptically and one piece (0.5 cm. x 0.5 cm. x 1.0 cm.) added to a tube

of nutrient broth and incubated several days to control for contamination. Tubes of both carrot and turnip were inoculated in duplicate; one set was incubated at 37°C and the other at 20°C. The progress of the rot was tested by probing with a stiff nichrome needle. Those showing active rot became soft and soon broke up. Unaffected pieces and controls in uninoculated broth were firm after weeks of incubation. This method proved far more satisfactory than inoculating sterile slices of carrot or turnip. This test is in reality a test of protopectinase production (Davison and Willaman, 1927).

The ability to macerate the vegetable tissue was possessed by sixteen of the *Erwinia* isolates (table 2). Five of the cultures (CA1, CA2, EC1, 495 and WV6) failed to act on the carrot, while three (CA1, EC1 and CA2) also had no effect on turnip. Inasmuch as all of the cultures were known at one time to be pathogenic for carrot and turnip, the ability to rot must have been lost in storage. The lower temperature was more effective, although some organisms were able to act on the middle lamella of the cells at 37°C.

Fifty coliform organisms including *Aerobacter aerogenes*, *A. cloacae*, *Escherichia freundii*, and *E. coli*, as well as intermediates, isolated from soil, grain, feces, etc., were tested similarly. In no case, however, was the slightest degree of maceration detectable (table 2). These tests were examined daily for three weeks and in each case the vegetable tissue remained as firm as the controls in uninoculated broth.

DISCUSSION

Had the *Erwinia* cultures used in this work been isolated in the course of a coliform investigation, there seems little doubt but that most would have been classified as aberrant or irregular coliforms. Without the aid of maceration experiments, there is no possible means by which they could be classified elsewhere. Classifying these organisms according to Stuart's aberrant coliform grouping, six are micro-aerogenic, while the others are anaerogenic. It is known, however, that, like coliforms, *Erwinia* cultures can shift from an anaerogenic state to an aerogenic one, or vice versa (Stanley 1939, Elrod 1941). It was originally contended by Harding and Morse (1909) that the anaerogenic soft-rot organisms were only strains of the aerogenic forms, and most certainly Dowson's (1941) recent separation of *Erwinia aroideae* and *E. carotovora* will break down due to this fact. Thus, the aerogenic and anaerogenic states in both the coliform group and *Erwinia* are not constant.

By means of the IMViC reactions plus cellobiose fermentation, *Erwinia* isolates can be placed in well-known coliform groups. Nine of the cultures were either group 8, $-+-++$, or group 9, $-+-+$A (Stuart's classification); four were in groups 2 and 3, $--+++$ and $--++$A; one was group 11, $-+--+$; one was group 12, $-+--$A; while four were grouped as irregulars, $-++++$ or $-+++$A. The majority of these fall into the group classified by Stuart as intermediates; none showing any apparent relationship to the *Escherichia coli* group. From this work it seems that the majority of the soft-rot organisms more nearly approached the characteristics of *E. freundii*, $-+-++$.

Gelatinolysis occurs more rapidly with *Erwinia* than with *Aerobacter cloacae*, at times the speed of reaction more nearly approaching the *Proteus* group. The fact that all of our *Erwinia* cultures are motile would likewise link the group with *A. cloacae*. It has also been shown by Elrod (1941) that a large majority of *Erwinia* strains ferment glycerol with the formation of acid only, seldom acid and gas. This also is characteristic of *A. cloacae*. The closer affinity of the soft-rot group would seem, therefore, to lie with *A. cloacae*, and not *A. aerogenes*. Depending on the criteria used, it would be possible to link *Erwinia* with either *Escherichia freundii* or *A. cloacae*.

The three pectic substances associated with plant tissues are protopectin, pectin and pectic acid. It is the protopectin which forms the cementing material of the middle lamella of plant cells. On hydrolysis protopectin yields an araban and pectin, and the binding property of the substance is lost. The separation of the cells from one another is usually spoken of as maceration. Macerating can be effected in many ways: long boiling (especially under pressure), treatment with 0.5 per cent ammonium oxalate at 70°–80°C, heating with dilute acids, electrodialysis and by the enzyme protopectinase. It is the latter which gives effectiveness to the soft-rot organisms and to certain fungi. Pectin fermentation, on the other hand, is undoubtedly due to several enzymes; pectinase, which hydrolyzes pectin and pectic acid to sugars and galacturonic acid, and the enzymes which would act on these sugars. It is apparent, therefore, that there is no reason to expect that the ability to ferment pectin is any indication of an invasive nature. From the experiments conducted here, it is obvious that coliform organisms do not possess the ability to liberate protopectinase and therefore cannot assume the role of attacking plants. This protopectinase activity would seem to be a criterion of sufficient importance to separate virulent *Erwinia* isolates from other lactose fermenters. It is difficult, however, to determine the taxonomic position of soft-rot organisms which have lost the ability to produce protopectinase. It seems, at present, that the ability to rot living plant tissue is a characteristic which warrants maintaining the soft-rot organisms in a position distinct from coliform bacteria, i.e., in the genus *Erwinia*. At the same time, however, one must recognize the very close relationship of the two groups. It is also true that such descriptions as "usually attack pectin" as given in Bergey's manual are entirely misleading.

CONCLUSIONS

The manner in which species of *Erwinia* ferment lactose would tend to classify them as aberrant coliforms. On the basis of their IMViC patterns, the majority would seem to approximate *Escherichia freundii*. On the other hand, according to their gelatin-liquefying ability, motility and production of acid in glycerol, the relationship seems to be closer to *Aerobacter cloacae*.

Sixteen of the *Erwinia* cultures possessed the ability to macerate vegetable tissue. This characteristic was not shared by any of the 50 coliform cultures. All but two of the *Erwinia* isolates fermented pectin in a synthetic medium, while 22 of the 50 coliform organisms, including three *Escherichia* of fecal origin, did so. There was no correlation between the ability to disorganize plant

tissue and pectin fermentation. On the basis of this macerating ability, it is contended that the placing of soft-rot organisms in a genus (*Erwinia*) separate from the coliforms is valid. At the same time the close relationship of the two groups is recognized.

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REFERENCES

BERGEY ET AL. 1939 Manual of determinative bacteriology. 5th ed. Baltimore.

BONDE, R. 1939 Comparative studies of bacteria associated with potato blackleg and seed-piece decay. Phytopathology, **29**, 831–851.

DAVISON, F. R., AND WILLAMAN, J. J. 1927 Biochemistry of plant diseases. IX. Pectic enzymes. Botan. Gaz., **83**, 329–361.

DOWSON, W. J. 1939 On the systematic position and generic names of the gram negative bacterial plant pathogens. Zentr. Bakt. Parasitenk. II., **100**, 177–193.

DOWSON, W. J. 1941 The identification of the bacteria commonly causing soft-rot in plants. Ann. Applied Biol., **28**, 102–106.

ELROD, R. P. 1941 Serological studies of the Erwineae. II. Soft-rot group; with some biochemical considerations. Botan. Gaz., **103**, 266–279.

HARDING, H. A., AND MORSE, W. J. 1909 The bacterial soft-rot of certain vegetables. I. The mutual relationships of the causal organisms. N. Y. Agr. Exp. Sta. Tech. Bull. 11.

JONES, L. R. 1905 Cytolytic enzyme produced by *B. carotovorus* and certain other soft-rot bacteria. Zentr. Bakt. Parasitenk. II., **14**, 910–940.

MCFADDEN, D. B. 1941 A chemical method for the sterilization of pectin. (Abst.) J. Bact., **42**, 289–290.

PARR, LELAND W. 1939 Coliform bacteria. Bact. Revs., **3**, 1–47.

STANLEY, A. R. 1939 Physiologic and serologic studies of the soft-rot and colon group of bacteria. W. Va. Agr. Exp. Sta. Bull. 287.

STUART, C. A., GRIFFIN, A. M., AND BAKER, MURIEL. 1938 Relationships of coliform organisms. J. Bact., **36**, 391–410.

STUART, C. A., MICKLE, F. L., AND BORMAN, E. K. 1940 Suggested grouping of slow lactose fermenting coliform organisms. Am. J. Pub. Health, **30**, 499–508.

STUART, C. A., BAKER, MURIEL, ZIMMERMAN, ALICE, BROWN, CAROLYN, AND STONE, C. M. 1940. Antigenic relationships of the coliform bacteria. J. Bact., **40**, 101–142.

YEASTS OCCURRING IN SOURING FIGS

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Figs frequently undergo an internal yeast fermentation while maturing on the tree. This is possible because botanically the fig is a syconium; a more or less hollow receptacle the inner walls of which are lined with flowers when immature and by seed-like fruits when ripe. It has an opening at the flattened end which is closed by overlapping scales during the early stages of development. After the fruit begins to ripen these scales loosen and an opening termed the "eye" is formed. At full maturity the flesh of each floret becomes juicy, forming an ideal medium for the growth of microorganisms carried into the fruit by various agencies, particularly by insects entering through the "eye". Beneficial as well as harmful insects may enter the fruit since the fig "wasp" (*Blastophaga psenes* L.) is necessary for pollination of the Calimyrna variety. This insect, however, as well as the undesirable ones may be responsible for infection of the fruit with destructive microorganisms. Fungi, other than yeasts and bacteria, causing fig spoilage have been studied extensively by Caldis (1927), Smith and Hansen (1931) and Hansen and Davey (1932). Most authors discussing fig spoilage refer to yeasts in connection with a type of deterioration termed "souring"; a spoilage involving the production of acid and a vinegar-like odor. Smith and Hansen (1927) described souring as a form of spoilage causing the contents of ripe figs to ferment and sour with subsequent dripping of liquid from the "eyes". Condit (1941) stated in regard to souring; "this is the cause of an immense loss of figs every year, especially in the Adriatic and Calimyrna varieties. Souring is caused by the action of specific yeasts and bacteria on the internal saccharine juice of the fig. Investigation has shown that figs are internally sterile until they are entered by insects, after which they commonly become infected with yeasts, molds, and bacteria." Caldis (1930) indicated that fig souring is primarily an alcoholic fermentation but subsequent changes may take place, the commonest being that brought about by the action of acetic acid bacteria on the alcohol with the production of acetic acid. Caldis isolated three types of yeasts, two of which produced typical souring when inoculated into ripe figs; the third type produced a different form of disease. The first two were termed *Mycoderma* and *Apiculata* and the third *Torula*. Davey and Smith (1933) included *Mycoderma*, *Pseudosaccharomyces*, *Hansenia*, and *Pichia* in the true souring yeasts of figs. The non-souring yeasts included those forming membranous, wrinkled, dry, surface growth on solid media. No other publications are available concerning the yeasts occurring in "souring" figs. It is apparent that the information available on these yeasts is meager and useless from the standpoint of the currently accepted taxonomy of yeasts.

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EXPERIMENTAL PROCEDURE

Thirty samples, each containing several souring figs were obtained in 1938 and 1939 from trees growing in widely separated areas in California. Most of the samples were collected in 10 different fig-producing districts located in the San Joaquin Valley, although some were obtained from an isolated orchard in Diablo Valley and others from three remotely located trees in the Santa Clara Valley. The latter two valleys are about 30 and 100 miles distant, respectively, from the centers of commercial production in the San Joaquin Valley.

The varieties collected included the Calimyrna which is insect pollinated and the Adriatic and Kadota which require no pollinization.

Isolation was accomplished by direct plating from the interior of infected fruits, all of which yielded yeasts. Identification procedures were similar to those employed by Mrak, Phaff, and Vaughn (1943). Qualitative tests for acid formation were made by observing for the clarification of slants of yeast infusion agar containing $\frac{1}{2}$ per cent of chalk, and of slants of 10° Balling fig extract agar also containing $\frac{1}{2}$ per cent chalk. For the quantitative determination of acid production 50 ml. of 20° Balling extract of dried figs were sterilized in 4 oz. bottles, then inoculated and stored 10 days at room temperature. Analyses were then made for total and volatile acids by the methods of the Association of Official Agricultural Chemists (1936).

To determine the tolerance to high concentration of sugars all yeasts isolated were inoculated in 30° Balling fig syrup. If growth occurred the cultures were transferred subsequently to 40° and finally 50° Balling syrup.

EXPERIMENTAL RESULTS

One hundred and fifteen isolates of yeast were obtained from 30 samples of souring figs. Sixty-four were sporulating and 51 non-sporulating forms. Most isolates proved to be species of *Saccharomyces* or *Candida*. Several isolates of *Pichia*, *Hanseniaspora*, *Kloeckera* and *Torulopsis* were also found. Of *Zygosaccharomyces*, *Zygopichia*, *Hansenula* and *Debaryomyces*, only single representatives were obtained.

Sporulating yeasts. Genus *Saccharomyces*: Thirty-six out of 37 of these organisms isolated were members of the subgenus *Saccharomyces* s.s. and only 1 of the sub-genus *Zygosaccharomyces*. Twenty-five of the isolates of *Saccharomyces* were *S. cerevisiae Hansen*, 6 *S. tubiformis* Osterwalder, 2 *S. fragilis* Jörgensen and 1 each of *S. cereviseae* var. *ellipsoideus* (Hansen) Dekker, *S. carlsbergensis* var. *monacensis* (Hansen) Dekker, and *S. carlsbergensis* var. *polymorphus* Dekker. The isolation of a single culture of *S. cerevisiae* var. *ellipsoideus* as compared with 25 of *S. cerevisiae* compares well with the results of Mrak and McClung (1940) in a study of yeasts occurring on California grapes.

The six isolates of *S. tubiformis* fermented maltose so slowly that gas formation could be determined only by use of the van Iterson-Kluyver fermentometer. Durham tubes and modified Durham tubes using 25 mm test tubes and 5 ml vials as suggested by Henrici (1941) gave negative results.

S. fragilis Jörgensen is a lactose-fermenting yeast that has been isolated com-

monly from dairy products but not previously from fruits. The isolates defined as *S. fragilis* differ from the type species in the three following respects: 1. Lactose is fermented only after adaptation to this sugar and then at a slower rate than by the type species; 2. Adaptation occurs only when the cells multiply in the presence of lactose and not simply by standing, while in a nonproliferating condition, in a medium containing this sugar; 3. One of the isolates was able to respire and assimilate maltose but could not ferment it; a phenomenon discussed in detail by Kluyver and Custers (1940).

The metabolic activities of these organisms and of the type species of *S. fragilis* were compared by use of Durham fermentation tubes, van Iterson-Kluyver fermentometers and the Warburg manometric technique. Multiplication of cells is facilitated in Durham tubes because of aerobic conditions. Adaptation occurs during this multiplication, hence the cells will ferment lactose irrespective of the medium on which previously grown. The rate of fermentation, however, is faster if the cells are taken from yeast infusion lactose slants. In the van Iterson-Kluyver fermentometer, multiplication of cells is practically absent because of the strictly anaerobic conditions. In view of this fact, scarcely any fermentation is observable if the cells are taken from malt agar slants. Cells taken from yeast infusion lactose slants show a slow fermentation requiring about 60 hours for completion. The type species on the other hand ferments a 4 per cent lactose solution to completion in 12–18 hours affected only slightly by the medium on which the cells were previously grown. These results show that if the fermentometer is to be used to test for the fermentation of specific sugars, the yeast should also be grown in a medium containing the sugar to be tested as the sole source of carbon. The results obtained by use of the Warburg manometric technique were similar but more exact from a quantitative point of view. The cultures isolated from figs showed neither respiration nor fermentation of lactose when grown on malt agar plates. When the cultures tested were grown on yeast-infusion lactose agar plates for 48 hours at 30°C. the rate of fermentation of the type species of *S. fragilis* was about five times that of the cultures isolated from figs. The rate of respiration on the other hand was of the same order of magnitude for all three cultures. The ability to ferment lactose is possessed by relatively few yeasts. To our knowledge no other species have been reported to require lactose adaptation before being able to ferment this sugar.

One of the lactose-fermenting yeasts isolated from figs showed the ability to assimilate maltose on an auxanogram plate. This was checked quantitatively by use of the Warburg technique. Anaerobic fermentation did not occur but there was appreciable respiration and slight aerobic fermentation (fermentation in the presence of air) of this sugar. The second culture isolated from figs as well as the type species was unable to attack maltose. Kluyver and Custers made a similar observation with *S. fragilis*. Other examples of this type of respiration and assimilation of disaccharides by yeasts which do not ferment these sugars have been given by Kluyver and Custers (1940) and Mrak *et al.* (in press). Kluyver and Custers explain this phenomenon by assuming a reversible inactivation of the hydrolases for certain disaccharides, under anaerobic conditions. They reject the possibility of direct respiration of the disaccharide.

Although the morphological characters of all these cultures are similar, the physiological differences between those isolated from figs and the type species of *S. fragilis* might appear to warrant the specific or varietal segregation of these organisms. However, cultures of *S. fragilis* sporulate freely and rapidly with subsequent discharge of the spores by rupturing of the ascus. This permits the mixing and fusion of spores from different cells with possible development of homozygous and heterozygous strains which may account for the physiological differences discussed above. In view of this it is advisable to term the isolates *S. fragilis* until single spore culture studies are made to determine whether or not the differences in fermentation and respiration result from hybridizations in nature.

The isolate of *S. carlsbergensis* var. *monacensis* agrees with the description of Stelling-Dekker (1931) but the variety *polymorphus* differs by forming slant cultures with entire rather than hairy borders. This species which ferments raffinose completely has been isolated in most instances from juices and fermenting liquors although it has been obtained occasionally from California grapes, dates, figs and prunes.

The subgenus *Zygosaccharomyces* was represented by a single culture of *Z. globiformis* Kroemer and Krumbholz which was similar in all respects to one previously isolated from California dates (Mrak *et al.*).

The isolation of a large number of *Saccharomyces* and only 1 *Zygosaccharomyces* from figs differs markedly from the proportion of these yeasts found on dates by Mrak *et al.* and on dried prunes by Mrak and Baker (1939). The yeast flora of figs resembles more closely that of fresh grapes, (Mrak and McClung, 1940).

The different fruits mentioned above vary considerably in sugar content. Fresh figs contain 20 to 35 and grapes 20 to 25 per cent of sugar. Dates, on the other hand, contain 65 to 70, dried figs 50 to 65, and dried prunes 40 to 50 per cent of sugar. Since most species of *Zygosaccharomyces* can grow in the presence of higher concentrations of sugar than other yeasts, the sugar content of the fruit is undoubtedly a factor influencing the relative prevalence of these 2 subgenera.

Genus *Pichia:* Sixteen isolates were included in the subgenus *Pichia* s.s. and 1 in *Zygopichia*. Fourteen of these were similar to *P. kluyveri*, an organism recently described by Bedford (1941). It is characterized by the fermentation of glucose, fructose and mannose, poor growth in synthetic medium, inability to utilize asparagin, ammonium sulfate, urea and nitrate, and by poor growth in alcohol medium without film formation, but with ester production. *P. kluyveri* differs from *P. fermentans* Lodder by failing to utilize asparagine, ammonium sulfate and urea; by the production of esters and by the absence of a pellicle in alcohol medium.

The production of esters is typical for the genus *Hansenula*, while unknown for species of *Pichia* until the isolation of *Pichia kluyveri*. It may be well to discuss in some detail the gradually fading borderline between the genera *Hansenula* and *Pichia*. Until the publication of Stelling-Dekker's monograph in 1931 the former genus differed from the latter by ascospore characteristics, its fermentative power, the formation of esters, the ability to split esculin and to utilize nitrate as the single source of nitrogen.

To differentiate the genera *Pichia* and *Hansenula*, the older literature laid considerable stress on spore morphology. Kloecker (1923), for example, defines the genus *Pichia* as having spherical, hemispherical, irregular or angular spores and *Hansenula* (*Willia*) as having hat- or saturn-shaped spores. However, Stelling-Dekker (1931) stated that both genera have hat-shaped spores in common, which also has been observed by the writers although the detailed morphology is somewhat different. *Pichia* spores usually have an oil droplet, but so have the spores of *Hansenula saturnus*. The authors are disinclined to believe that the spore morphology forms a strict character for generic differentiation. In 1932 Lodder published the description of *Pichia fermentans*, which broke down the fermentation point of difference. In 1941 Bedford described *Pichia kluyveri*, able to produce at least as much ester as some *Hansenula* species and also able to split esculin. This left as the only point of difference the utilization of nitrate, because morphologically the two genera are very similar. Bedford (1941) and also Mrak *et al.* found a species of *Hansenula*, termed *H. subpelliculosa*, which does not attack nitrate in a completely synthetic medium in contrast to all other *Hansenula* species. The auxanogram plate is positive probably because the heavy inoculum introduces enough growth substances to permit development at the expense of the nitrate added.

To differentiate two genera on a single physiological character of a nature as described seems unsound to us. It may be advisable in the near future to form one genus, containing subgenera of the nature of *Pichia*, *Zygopichia*, *Hansenula* and *Zygohansenula*.

A single organism was considered to be *P. fermentans* Lodder although there are differences in film, slant culture and spore characteristics. The film produced is thin and smooth, the slant culture dull, and ascospores spherical rather than hat-shaped. Such differences might be considered sufficient for species segregation but in our experience these characters show considerable variation in the genus *Pichia*. Slant cultures and films frequently undergo variations in response to the particular conditions of growth and ascospores may vary in shape from spherical to hemispherical or be slightly hat-shaped in the same species.

One isolate of *Pichia belgica* (Lindner) Dekker was found, which agrees quite well with the description of the type species by Stelling-Dekker. In view of the fact that this worker was unable to obtain ascospores with the type species present in the collection of the Centraalbureau voor Schimmelcultures at Baarn, Holland, it is worthwhile mentioning that our culture sporulated abundantly on any type of medium. The morphology of the spores is similar to the original description and drawings given by Lindner (1909), that is, indistinctly hat-shaped with a short brim.

The isolate of *Zygopichia* is similar to *Z. chevalieri* (Guilliermond) Dekker.

The occurrence of fermenting species of *Pichia* has been reported relatively few times. Aside from *P. fermentans* Lodder all isolations of fermenting species of *Pichia* have been made in California. *P. kluyveri* has been obtained from figs and pickled olives, *P. chodati* var. *fermentans* from dates, and *P. fermentans* from figs.

Genus *Hanseniaspora:* Eight isolates, similar in all respects to *H. melligeri*

Lodder, were obtained from eight different fig samples. This is one of the commonest species of *Hanseniaspora* in California. Besides Melliger's isolate (1931) from Egyptian dates, we have obtained this organism from California dates, figs, prunes and apples from widely separated areas. Except for the description by Lodder (1932) no other reports of the occurrence of this organism have been found.

Genus *Hansenula:* A single isolate of *H. anomala* var. *sphaerica* (Naegeli) Dekker was obtained. Relatively few cultures of *Hansenula* have been reported from California fresh fruits. Several cultures, on the other hand, have been isolated in California from beverages, concentrates, pickles and stored foods such as dried fruits.

Genus *Debaryomyces:* The single isolate obtained differs from described species in its ability to ferment maltose, growth in alcohol medium and in cell size. The fermentation of maltose is slow but is easily detectable in Durham tubes. The culture isolated from figs is described as *D. dekkeri.*

Debaryomyces dekkeri nov. sp. Cells spherical to globose in 1, 3, and 40 day liquid wort cultures. Cells in 1 and 3 day liquid wort cultures range (2.4–3.6μ) x (2.4–3.6μ) and average (3 x 3μ). No pellicle formation in 40 days but a narrow ring appears in 5 days. In young cultures cells single or in pairs; clusters present in 40 day cultures. Budding on all sides. Ascospore formation follows iso. or heterogamic conjugation. Ascospores spherical and rough with centrally located oil droplet. One or 2 ascospores form in one of the conjugating cells. Ascospore size averages (2.9 x 2.9μ). Cells sometimes form abortive conjugation tubes. Ferments glucose, fructose, mannose, sucrose, maltose slowly and $\frac{1}{3}$ of raffinose. Does not ferment galactose or lactose. Utilizes asparagin, ammonium sulfate, urea and peptone but not nitrate. A good growth in alcohol medium. Slant culture pale olive buff, smooth to slightly verrucose, glistening, and convex with entire borders.

Species of *Debaryomyces* apparently seldom occur on fruits or fruit products but commonly on pickles and meats (Mrak and Bonar 1938, 1939).

Non-sporulating yeasts. The majority of the non-sporulating yeasts were species of *Candida, Kloeckera* and *Torulopsis.*

Genus *Kloeckera:* Of 13 isolates belonging to this genus 12 were *K. lindneri* (Kloecker) Janke and 1 *K. africana* (Kloecker) Janke. The isolates of *K. lindneri* differed slightly from the organism described by Lodder (1934) in cell size and consistent inability to liquefy gelatin. The culture of *K. africana* produced esters and an incomplete pellicle of loose islets in wort. These characteristics apparently do not occur in the type culture of *K. africana.*

Sporulating as well as non-sporulating apiculate yeasts have been isolated from fresh fruits in California but only the sporulating genus *Hanseniaspora* from dried fruits. The concentration of sugar present in dried fruits may be considered a factor favoring sporulation or inhibiting the growth of *Kloeckera.* However, the isolates of *Kloeckera* and *Hanseniaspora* from figs showed no difference in tolerance to high concentration of sugar. Both grew in 40°, but not in 50° Balling fig syrup. Cultures of *Hanseniaspora* isolated from dates on the other hand grew well in 50° Balling date syrup.

It is possible that the isolates of *Kloeckera* are in reality imperfects of *Hanseniaspora* that have lost their ability to sporulate. If this is true it may be assumed that the isolates of *K. lindneri* are non-sporulating forms of *H. melligeri*. The consistent ability of *H. melligeri* and inability of *K. lindneri* to liquefy gelatin might be explained by the observation of Beijerinck (1898), that sporulating cells of certain yeasts liberate the protoplasm not involved in spore formation, soon after this process has taken place. The liberated protoplasm can then cause liquefaction as soon as it comes in contact with the gelatin. According to Beijerinck, protoplasm is not liberated by non-sporulating strains until the cells die. Since this occurs more slowly than sporulation, a much longer time is required for the non-sporulating strain to liquefy gelatin if liquefaction occurs at all.

Genus *Torulopsis:* Six isolates of *T. stellata* (Kroemer and Krumbholz) Lodder were obtained from samples collected in four different districts. As far as can be determined this organism was previously isolated only in Germany, from the juice of partially vine-dried grapes (Trockenbeerenauslese).

Genus *Candida:* Thirty-three isolates of *Candida* were obtained, twenty-six of which were *C. krusei* (Castellani) Berkhout as described by Langeron and Guerra (1938). Six were considered to be *C. chalmersi* Castellani although maltose and raffinose were fermented slowly and detectable only by use of the van Iterson-Kluyver fermentometer. The maltose auxanogram was definitely positive. *C. chalmersi*, according to Langeron and Guerra, ferments neither maltose nor raffinose but utilizes maltose in an auxanogram plate.

One isolate of *Candida* differs from the described species although it possesses characteristics of the *guilliermondi* group. It differs from *C. guilliermondi* by forming a dry, wrinkled rather than slimy film on wort, forming a film on alcohol medium and failing to ferment maltose, although utilizing it on an auxanogram plate. This isolate differs from *C. chalmersi* in pellicle formation on wort and alcohol medium and by fermenting galactose, sucrose, and slowly, $\frac{1}{3}$ of the raffinose. This organism is not an imperfect form of *Hansenula* because of its inability to utilize nitrate, nor of *Endomycopsis* because of the absence of true septa. However, until Diddens and Lodder complete their monograph of the genus *Candida* this yeast will be included in the group *guilliermondi*, without specific designation.

The isolation of such large numbers of *Candida* from California figs, dates and grapes indicates that these organisms are rather wide-spread in nature. The isolates from figs and dates have been in most instances *C. krusei* and *C. chalmersi*. Relatively few or none of the other species have been found on these fruits. It is of interest that thus far *C. albicans* has not been encountered in our yeast flora studies.

Distribution. The commonly isolated yeasts such as *S. cerevisiae*, *H. melligeri*, *C. krusei* and *C. chalmersi* were obtained from all varieties of figs tested.

The more common yeasts were found in all of the important fig producing areas. *S. cerevisiae* was found in all and *C. krusei* in 8 of the 10 districts in which samples were collected. The single isolates of *H. anomala* var. *sphaerica* and *Z. globiformis* were from fruit grown in Santa Clara Valley which is located about

100 miles from the center of fig production. *Candida* species, on the other hand, were not obtained from figs produced in this area even though several samples were collected at various times during two seasons. All of the commonly isolated yeasts were obtained in both 1938 and 1939.

Tolerance to high concentrations of sugar. Most of the yeasts isolated from figs grew in 40° but not 50° Balling fig syrup. Thirteen cultures of *S. cerevisiae*, 7 of *C. krusei* and 1 each of *Zygopichia chevalieri* and *P. belgica* failed to grow in 40° Balling fig syrup. The cultures of *C. chalmersi* and *Z. globiformis* were the only organisms showing even slight growth in 50° Balling fig syrup. These yeasts are considerably less tolerant to high concentrations of sugar than those found on dates.

Acid production. Soured figs have been given this designation primarily because of the presence of a distinct acetic acid taste and odor. In fact, an organoleptic procedure is used by dried fig inspectors to determine the presence or absence of souring. As indicated in an earlier paragraph, many of the authors discussing fig spoilage have indicated the direct or indirect connection of yeasts with fig souring. These circumstances prompted the present authors to culture the yeasts on Custer's yeast-water-glucose-chalk and fig-infusion-chalk agar slants. Thirty-six isolates showed at least a slight clearing of these chalk containing media. *H. anomala* var. *sphaerica* and *K. africana* clarified the slants much more rapidly than any of the others. Quantitative tests were made for volatile acid and fixed acid production with the thirty-six isolates causing clarification of the chalk agar. The results are summarized in table 1. It is apparent from the data given that volatile and fixed acid production was low in most instances and not sufficient to cause the spoilage termed souring. In addition, members of the two genera *Saccharomyces* and *Candida* which were represented most abundantly produced least acid of all, while *H. anomala* and *K. africana* were only represented by single isolates. Experiments with acetic acid bacteria

TABLE 1

*Acid produced by different yeasts in fig extract**

ORGANISM	NUMBER OF ISOLATES TESTED	ACID PRODUCED IN 10 ML. AS ML. OF N/10 NaOH		
		Total	Volatile	Non-volatile
P. kluyveri	11	2.83–5.50	0.24–0.60	2.59–9.40
H. melligeri	8	3.18–5.30	1.25–2.65	1–93–2.65
C. chalmersi	6	0.69–1.88	0.0 –0.05	0–69–1.83
K. lindneri	3	3.78–4.03	1.30–3.02	2.48–1.01
C. krusei	2	0.0 –1.97	0.0 –0.05	0.0 –1.92
S. cerevisiae	2	3.94–3.95	0.25–1.11	2.84–3.69
T. stellata	2	3.18–4.63	0.29–0.44	2.89–3.19
K. africana	1	3.77	1.25	2.52
H. anomala var *sphaerica*	1	8.60	6.30	2.30

* When determinations were made with more than 1 culture of a given species the range of acid production is given.

isolated from figs showed that these organisms alone produce appreciable quantities of fixed acid but only traces of volatile acid in fig infusion medium which cannot account for the acetification observed in fig spoilage, whereas heterofermentative lactic acid bacteria produced appreciable quantities of volatile acid. However, only a few cultures of *Lactobacillus* and *Leuconostoc* were encountered whereas cultures of *Acetobacter* were isolated from all figs examined. Most of the fig souring apparently results from the associative action of yeasts and acetic acid bacteria.

SUMMARY

One hundred and fifteen yeasts were isolated from 3 varieties of souring figs· Most of the yeasts isolated were species of *Saccharomyces* or *Candida*. The species of *Saccharomyces* in order of importance were *S. cerevisiae* (25), *S. tubiformis* (6), *S. fragilis* (2) and 1 each of *S. cerevisiae* var. *ellipsoideus*, *S. carlsbergensis* var. *monacensis* and *S. carlsbergensis* var. *polymorphus*. The *Candida* species were *C. krusei* (26), *C. chalmersi* (6), and an unidentified species. Other organisms were *Pichia kluyveri* (14), *P. fermentans* (1), *P. belgica* (1), *Zygospichia chevalieri* (1), *Hanseniaspora melligeri* (8), *Kloeckera lindneri* (12), *K. africana* (1), and *Torulopsis stellata* (6), and single isolates of *Zygosaccharomyces globiformis*, *Hansenula anomala* var. *sphaerica*, and a new species of *Debaryomyces*.

The sugar tolerance of the organisms isolated was low; most of them growing in 40° but not 50° Balling fig syrup. The production of volatile and fixed acids was low and not sufficient to cause the spoilage termed souring.

Evidence is presented to show that adaptive lactase formation occurs in organisms termed *S. fragilis*.

REFERENCES

Association of Official Agricultural Chemists 1936 Official and Tentative Methods of Analyses. Washington, D. C. 4th ed.

Bedford, C. L. 1941 Genus Hansenula Sydow. Morphological and Physiological Studies. Ph.D. Thesis, University of California at Berkeley, 1–128.

Beijerinck, M. W. 1898 Sur la régénération de la faculté de produire des spores chez des levures en voie de la perdre. Arch. néerland. sci. Série II, Tome II.

Caldis, P. D. 1930 Souring of figs by yeasts and the transmission of the disease by insects. J. Agr. Research, **40**, 1031–1051.

Caldis, P. D. 1927 Etiology and transmission of endosepsis (internal rot) of the fruit of the fig. Hilgardia **2**, 287–328.

Condit, Ira J. 1941 Fig culture in California. Calif. Agr. Ext. Circ., **77**, 1–67.

Custers, M. Th. J. 1940 Onderzoekingen over het gistgeslacht Brettanomyces. Diss. Delft.

Davey, A. E., and Smith, Ralph E. 1933 The Epidemiology of Fig Spoilage. Hilgardia, **7**, 523–551.

Hansen, H. N., and Davey, A. E. 1932 Transmission of smut and mold in figs. Phytopathology, **22**, 247–252.

Henrici, Arthur T. 1941 The yeasts: genetics, cytology, variation, classification and identification. Bact. Revs., **5**, 97–179.

Klöcker, Alb. 1924 Die Gärungsorganismen. Dritte Aufl. Urban & Schwarzenberg, Berlin-Wien.

KLUYVER, A. J., AND CUSTERS, M. TH. J. 1940 The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. Antonie van Leeuwenhoek, **6**, 121-162.

LANGERON, M., AND GUERRA, P. 1938 Nouvelles recherches de zymologie médicale. Ann. parasitol., **16**, 36-84, 162-179, 481-525.

LINDNER, R. 1909 Mikroskopische Betriebskontrolle in den Gärungsgewerben. Fünfte. Aufl., Paul Parey, Berlin.

LODDER, J. 1932 Über einige durch dass "Centraalbureau voor Schimmel-cultures" neuerworbene sporogene Hefearten. Zentr. Bakt. Parasitenk., II, **100**, 227-253.

MELLIGER, R. 1931 Contribution à l'étude des ferments figurés et les fermentations de la datte. Thèse No. 893. Genève.

MRAK, E. M., AND BAKER, E. E. 1939 Sugar tolerant yeast causing the spoilage of dried fruits. Third International Congress for Microbiology. New York, 707-708.

MRAK, E. M. AND BONAR, LEE 1938 A note on yeast obtained from slimy sausage. Food Research **3**, 615-618.

MRAK, E. M. AND BONAR, LEE 1939 Film yeast from pickle brines. Zentr. Bakt. Parasitenk, II, **100**, 289-294.

MRAK, E. M. AND McCLUNG, L. S. 1940 Yeasts occurring on grapes and in grape products in California. J. Bact., **40**, 395-407.

MRAK, E. M., PHAFF, H. J. AND VAUGHN, R. H. 1942 Yeasts occurring on dates. J. Bact., **43**, 689-700.

SMITH, RALPH E. AND HANSEN, H. N. 1927 The improvement of quality in figs. Univ. Calif. Agr. Expt. Sta. Circ., **311**, 1-23.

SMITH, RALPH E., AND HANSEN, H. N. 1931 Fruit spoilage diseases of figs. Calif. Agr. Expt. Sta. Bull., **506**, 1-84.

STELLING-DEKKER, N. M. 1931 Die Hefesammlung des "Centraalbureau voor Schimmelcultures." 1 Teil. Die Sporogenen Hefen. Verhandel. Akad. Wetenschappen Amsterdam. Afdeel. Natuurkunde. 2 sectie. Deel 28 No. 1.

STUDIES ON THE LUMINOUS BACTERIA

I. Nutritional Requirements of Some Species, with Special Reference to Methionine

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Anyone who has had occasion to isolate luminous bacteria can readily understand why these organisms have received so much attention from microbiologists of the present and of the last centuries The aesthetic satisfaction derived from the contemplation of these beautiful organisms repays the investigator many fold for the hours spent in darkness and seclusion *vis a vis* some fetid putrefying fish. Due partly, no doubt, to this fact, a voluminous literature dealing with these bacteria has accumulated. For an excellent bibliography and a review of all the most important contributions to the knowledge of the luminous bacteria and to the understanding of the physiology of luminescence, reference need only be made to Pratje (1923) and Harvey (1940, 1941).

Owing to the recognition that the light-emitting process is a by-path of respiration, and to the convenience of using changes in the intensity of luminescence as an indicator of physiological events in the organisms, the luminous bacteria have acquired, in the last few years, a new importance in scientific research, as material for exceedingly interesting experiments on general physiological problems. (E.g., Johnson, 1938, 1939.) Little attention, however, has been devoted to the natural history of this group of bacteria since Beijerinck's studies (Beijerinck, 1889, 1912, 1916). Although innumerable species have been described, no thorough systematic study of the group has ever been undertaken with a view to clarifying their relationship among themselves or with other bacteria.

In the hope of contributing to the elucidation of some of the complex taxonomic and physiological problems involved in the sudy of this group of organisms and to the better understanding of some previously recorded observations on the behavior of the bacteria under certain conditions, a series of investigations has been carried out on the nutrition and metabolism of several species, the results of which seem to be of sufficient general interest to warrant their publication.

In the present paper, the results of experiments on growth requirements of a number of strains will be presented.

MATERIAL AND METHODS

The various strains of luminous bacteria used in the present studies were obtained as follows. A culture of *Photobacterium phosphoreum* and one of *Photobacterium sepiae* from Prof. A. J. Kluyver's collection at Delft, were kindly supplied by Dr. F. H. Johnson, as was the type strain of *Achromobacter harveyi*

and the strain identified by Dr. Johnson as *Photobacterium fischeri* and used by him in the redescription of that species (Johnson and Shunk (1936)). A culture of *Photobacterium splendidum*, probably representing the original, Beijerinck strain, was given to me by Dr. C. E. Clifton, who obtained it also in Delft. Beside the strain of *Photobacterium phosphoreum* already mentioned (to be referred to as the "Delft strain"), ten morphologically and physiologically similar strains were selected for experimentation from those isolated on various occasions at Pacific Grove and Berkeley, California. Of these, nine, including strain no. 2 and the strain used in previously reported experiments (Doudoroff, 1938) were isolated from decomposing flatfish and one (Strain 12) from dead squid.

The basal medium consisted of distilled water containing M/30 Sörensen KH_2PO_4-Na_2HPO_4 phosphate buffer mixture at pH 7.0; 3 per cent NaCl; 0.03 per cent NH_4Cl; 0.03 per cent $MgSO_4$; 0.001 per cent $FeCl_3$; 0.001 per cent $CaCl_2$; and 0.1–0.3 per cent of the organic carbon source by weight. No attempt was made to study in detail the mineral nutrition of the organisms, and traces of all essential elements were assumed to be present in the reagents used.

Synthetic organic compounds were used wherever possible. Glucose was autoclaved separately in distilled water, since it was found to give rise to exceedingly toxic products if autoclaved with phosphates. Thermo-labile compounds were sterilized separately by filtration through glass filters and added to the sterile basic medium.

The medium was dispensed in 4 ml. amounts in large pyrex test tubes to insure an adequate oxygen supply, no special means of aeration or agitation being used.

Transfers of the organisms from tube to tube were made with a platinum loop of 0.003 ml. capacity, except in experiments with *P. phosphoreum*, where 0.05 ml. of the culture was used for inoculation. This large inoculum was selected for three reasons. Firstly, the development of this species was quite slow in synthetic media, and a great deal of time could be saved by using an inoculum which would give rise to an earlier development of visible turbidity. Secondly, results obtained with smaller inocula were often inconsistent, due, apparently, to the very rapid senescence and autolysis of the bacteria, especially in carbohydrate-containing media, after the "maximum stationary phase of development" was attained. Thirdly, it appeared that in many cases an "adaptation" of the culture, involving the selection of individuals possessing special capabilities was necessary for the development of cultures upon transfer to a medium of a diffeıent composition from that to which the bacteria had become accustomed; and a larger inoculum would provide greater chances of obtaining "adaptable" cells (Doudoroff, 1940). Since the use of heavy inocula involves a great "carry-over" of any added substances from one culture to the next, no synthetic medium was regarded as satisfactory unless the bacteria could be cultured with it through at least seven serial transfers.

Whenever an attempt at substitution of one of the constituents of a satisfactory medium by another resulted in the failure of the organisms to develop upon initial transfer, the experiment was repeated, using a series of concentra-

tions of the new factor and, in some cases, different initial pH values of the basal medium. Furthermore, the bacteria were allowed to develop in a medium containing a satisfactory nutrient factor together with the one under investigation and again transferred to the altered medium. Such cultures not only served as tests for the possible toxicity of the new factor, but in some cases apparently favored the "adaptation" of the organisms to the new conditions. Tubes showing no development were usually kept for from fifteen days to a month before being discarded.

NUTRITION OF P. FISCHERI, P. SPLENDIDUM, P. SEPIAE AND A. HARVEYI

All of the above species developed readily in the synthetic basal medium with glucose as sole carbon source, with mean division times from 100 to 150 minute at 18–20°C. Growth was considerably slower with synthetic glycerol[1] in place of glucose, the division rate being approximately one third of that observed with glucose. On first transfer to synthetic medium with glycerol, *A. harveyi* developed still more slowly, but after two subcultures in this environment a constant rate of reproduction comparable to that found in other species was established. With alanine as the only substrate, *A. harveyi* propagated quite readily, *P. sepiae* and *splendidum* very slowly, and *P. fischeri* not at all.

Unlike *P. phosphoreum*, which always showed fairly good luminescence in synthetic media, the other species emitted very little light or none at all, retaining, however, their ability to luminesce upon return to complex media.

NUTRITION OF P. PHOSPHOREUM

In contrast to the other species studied, most strains of *P. phosphoreum* did not develop readily in the basal medium with a single carbon source.

Experiments with strain no. 2

Strain no. 2 was used for a series of investigations which were carried out over a two-year period. This strain did not propagate in the basal medium with either glucose, glycerol, or alanine as carbon source, unless a mixture of amino acids was also added. By the process of elimination, it was found that the effect of the mixture could be duplicated by the use of a single amino acid: dl-methionine. Methionine could not be replaced by any other single pure compound or any of several hundred combinations tried.

With glycerol as chief carbon source, distinct turbidity due to bacterial development could be detected when a concentration of methionine corresponding to 0.2 mg. per liter of culture medium was used. With higher concentrations, more growth was obtained, maximum development occurring with from 3 to 5 mg. per liter. Greater amounts (up to 20 mg. per liter) had neither a more favorable nor any unfavorable effect. Approximately the same concentrations of methionine were effective with glucose, lactate, succinate, fumarate, or alanine as chief carbon sources in place of glycerol.

It should be pointed out that ordinary samples of leucine, which contain

[1] Synthetic glycerol was kindly furnished by the Shell Development Company.

methionine as an impurity, could be used in place of methionine, as well as one sample of tryptophane (which theoretically should not be so contaminated). However, neither synthetic dl-leucine nor methionine-free l-leucine, nor other samples of tryptophane tested had any activity. An interesting complementary effect of leucine and methionine came to light in these experiments. Leucine, which, in itself, is apparently unsatisfactory either as carbon or nitrogen source to the organisms, in some manner "protected" methionine, so that the addition of 0.01 per cent dl-leucine to the basic medium with glycerol made it possible to obtain maximum growth with as little as 0.3 mg. of methionine per liter.

The following substances could not be substituted for methionine either alone or in any combination so far tried: sulfur, sulfide, sulfate, thiosulfate, thiourea, thioglycollate, cystine, S-methyl-cysteine, benzyl hemocysteine, homocystine, glutathione, thiamine, glycine, alanine, serine, threonine, α amino-n-butyric acid, valine, leucine, norleucine, isoleucine, aspartic acid, asparagine, glutamic acid, arginine, tyrosine, phenylalanine, tryptophane, proline, choline, as well as a number of organic acids, alcohols, and recognized growth-promoting substances.

Although the addition of mixtures of amino acids to the medium increased the growth rate of the organisms considerably, the same amino acids added singly often had a distinctly inhibitory effect. Thus, the addition of 2 mg. of homocystine or S-methyl-cysteine per liter of glycerol-methionine medium either considerably delayed or completely prevented development, whereas 5 to 10 mg. per liter could be easily tolerated if, in addition, a little peptone or a mixture of glycine, alanine, leucine and asparagine was added. α amino-n-butyric acid was inhibitory in even lower concentrations unless traces of peptone were present.

With methionine as accesory factor, glucose, glycerol, lactate, pyruvate, succinate, fumarate, alanine, aspartic acid and asparagine were found to be satisfactory carbon sources. On the other hand, formate, acetate, malate, glycine and leucine appeared to be unsatisfactory. Cultures developing with glucose and, to a lesser degree, with glycerol tend to become acid, and the organisms in such cultures show senescence and autolysis sooner than those developing with substrates such as organic acids and amino acids, the utilization of which usually results in the increased alkalinity of the medium.

When serial transfers were made in media with certain carbon sources, a very long "lag phase" preceding visible development of the bacteria was often encountered, even though the organisms were transferred to the same medium in which they had propagated. Indeed, occasionally, subcultures failed completely, although the bacteria were still luminous and viable in peptone media at the time of inoculation. Such behavior of the bacteria was most striking in media with pyruvate, lactate and fumarate as chief carbon sources, but was also observed in cultures with succinate, aspartic acid, and asparagine.

The lag period observed in such instances could be greatly reduced or eliminated if a larger inoculum was used or if a trace of glycerol was added to the fresh medium. It was further found that, in the case of cultures grown with pyruvate, the introduction together with the inoculum of some of the culture medium in which the bacteria had developed, freed of the organisms by centrif-

ugation or filtration, had a similar effect to that of using a large inoculum. The stimulating factor or factors were absent from the neutral distillate of such a medium but could be recovered in the steam-volatile acid fraction as well as in the residue after steam distillation. Since formic, acetic, lactic and succinic acids were found to be among the products of fermentation by *P. phosphoreum*, these compounds were tested for growth-promoting activity. Indeed, the addition of 0.002 per cent of sodium formate, acetate, lactate, succinate, or fumarate hastened development with pyruvic acid as chief carbon source. Varying the CO_2 partial pressure by the addition of bicarbonate to the medium, of CO_2 to the gas phase, or both, did not seem to affect the growth. With lactate as chief carbon source, fumaric, succinic and aspartic acids exhibited growth-stimulating properties, although not as marked as those of glycerol. To a lesser degree, pyruvic, lactic, formic, acetic, and aspartic acids stimulated development in a fumarate-containing medium.

Experiments with eight similar strains

Eight other strains of *P. phosphoreum*, tested for their ability to grow on synthetic media with and without methionine after more or less prolonged cultivation on peptone-glycerol agar, developed in the presence, but not in the absence of methionine. On first transfer to synthetic media, two of these developed with alanine and methionine but not with glycerol and methionine, two others grew in the latter but not in the former medium, while the other four developed with either carbon source supplemented with methionine. After developing in a synthetic medium, all strains were found to be capable of using both alanine and glycerol as chief carbon sources, but still unable to grow in methionine-free medium.

Experiments with strain no. 12

In contrast to the previously discussed strains, strain no. 12, a particularly hardy and rapidly growing culture of *P. phosphoreum*, was found to be capable of developing without methionine in the basal medium with glucose, glycerol, or alanine as sole carbon sources. However, in the first transfers to synthetic medium, methionine had a strikingly stimulating effect, which became somewhat less pronounced after prolonged cultivation in methionine-free media. With glucose as carbon source, it could be shown that transfers made from the logarithmic or the very early stationary phases of development were less affected by methionine than those made from the later stationary phase. In fact, if the transfers were made at a properly selected time, the organisms did not develop at all without added methionine, while as little as 0.1 mg. of this substance per liter was sufficient to initiate growth. Transfers made from the senescent phase could no longer develop in media with methionine unless peptone was also added.

An unexpected observation was made with one of the cultures of this strain that had been grown for some time with alanine as sole carbon source. Methionine, added to the alanine medium, was in some cases inhibitory to development

in the same concentrations in which it exerted a stimulating effect on the same culture transferred to media with glycerol or glucose. This inhibitory effect, demonstrated by a prolonged lag phase, was noted only if the transfers were made during the logarithmic phase or before maximum development had been reached and not in the case of transfers made from older cultures.

Experiments with the Delft strain

In the first attempts to cultivate the "Delft strain" of *P. phosphoreum*, in synthetic media, good growth was obtained in the basal medium with either glucose or glycerol (but not with alanine) as chief carbon source, provided methionine was also added. From such cultures, the organisms could be transferred to the alanine-methionine medium, in which the bacteria propagated readily. Attempts to cultivate the organisms in methionine-free media were generally unsuccessful, with a few interesting exceptions. As a rule, the amount of growth was found to depend on the concentration of methionine in a manner similar to that observed with strain no. 2. Also, as with strain no. 2, cystine, S-methyl-cysteine, glycine, alanine, leucine, asparagine, proline, choline and, in addition, creatine could not replace methionine. However, homocystine, which was apparently unsuitable to the other strain, was found to be a good substitute for methionine with the Delft strain, provided the bacteria were first allowed to grow in the basal medium containing glucose and both methionine and homocystine. Surprisingly, it was found that from the media in which homocystine was used in place of methionine, the bacteria could now be transferred to media containing glucose or glycerol alone, and could be cultivated indefinitely in such media without any accessory growth factors. The first transfers from homocystine-containing to homocystine-free media sometimes gave rise to imperfectly developing cultures, which showed but slight turbidity followed by rapid autolysis. However, transfers to the same medium from such abortive cultures at the peak of their development gave rise to vigorously growing sub-cultures. It must be added that after several unsuccessful attempts a single culture requiring no accessory factors was also obtained by heavily seeding a methionine-free medium containing alanine with bacteria from an alanine-methionine culture which had not yet reached the stationary phase of development. Growth was very slow at first, but after several transfers the rate of development increased. It therefore seems probable that cells not requiring any accessory factors occur occasionally in all cultures of the bacterium. A conceivable explanation for the "weaning" of the bacteria from accessory growth factors by the intermediate substitution of homocystine for methionine could be offered on the assumption that the latter compound serves several purposes to the organisms, not all of which (*e.g.*, source of methyl group) can be served by the former. An "adaptation" of the culture either through "acclimatization" of the cells or the selection of those least dependent on methionine might thus have been accomplished by cultivation in a partially deficient medium.

The cultures "adapted" to growth without accessory factors were benefited by the addition of methionine or homocystine. As with strain no. 12, the

growth-promoting effect of methionine was most pronounced if transfers were made from cultures in the stationary phase of development.

It here seems appropriate, although not altogether pleasant, to record some further observations on the somewhat paradoxical behavior of some cultures of this strain under certain conditions, which at present seem further to confuse the already somewhat complex picture of the growth requirements of the species.

Although the "Delft strain" could tolerate considerably higher concentrations of homocystine than strain no. 2, amounts greater than 5 mg. per liter of glucose methionine medium were found to be somewhat inhibitory to its development. A "detoxification" similar to that observed with strain no. 2 and a marked increase in the rate of growth could be accomplished by adding a mixture of small amounts of glycine, alanine, leucine, asparagine and proline. However, after the organisms had become accustomed to homocystine and this compound was substituted for methionine as the accessory factor, the same mixture of amino acids exhibited a somewhat retarding action on growth in glucose-homocystine medium.

As stated earlier, even after the "adaptation" of cultures to an existence in an environment containing neither methionine nor homocystine, the addition of these compounds stimulated their growth. Seemingly anomalous results, however, were occasionally obtained in experiments in which the effect of different concentrations of methionine on the development of such cultures was tested. Without added methionine, the cultures grew somewhat more slowly, but to as great an extent as those receiving 3 to 5 mg. of methionine per liter. Those, however, developing with low concentrations of methionine (0.1 0.5 mg. per liter) grew rapidly at first, but showed considerably less turbidity at the peak of development, the magnitude of the maximum crop depending on the concentration of the accessory factor. Although usually the bacteria underwent a rather rapid autolysis after the stationary phase was reached, on one occasion a rapid but slight development in a methionine-poor medium was followed at first by the usual stationary phase and partial autolysis, and then by a second period of growth, leading to turbidity as heavy as that obtained in cultures under optimal conditions. Two possible interpretations of the peculiar response of the bacteria to low concentrations of methionine, which seems to controvert the general belief that half a loaf is better than no bread at all, may be suggested: a) The addition of small amounts of methionine to the medium may result in the preferential propagation of cells incapable of growing without this factor and a subsequent change in the external environment due to the development of such cells, which, in turn, inhibits the multiplication of those cells for which methionine is unnecessary; or, more probably, b) The increased rate of development in a medium containing little methionine leads to the exhaustion or destruction of some internal mechanism in the cells which is necessary for propagation in the absence of the accessory factor. The lack of sufficient methionine, possibly coupled with some external changes brought about by the development of the culture, may then become limiting to growth. Somewhat

in contradiction to either of the above hypotheses was the result of a single experiment, in which a culture which had been adapted to growth without methionine was allowed to develop in the presence of a sufficient amount of this substance to permit maximum growth, and then transferred back to methionine-free medium, wherein profuse growth was observed.

DISCUSSION

In view of the demonstrated ability of at least some strains of all of the luminous bacteria studied to grow in synthetic media with a single carbon source, the distinction made by Beijerinck (1916) between types requiring peptone and those requiring both peptone and carbohydrate loses most of its significance. At first, a clear-cut physiological differentiation of *P. phosphoreum* from the other species studied seemed possible on the basis of its requirements for methionine, but the cases of adaptation of some strains to media without this compound have made clear the illusory nature of such criteria. The danger of relying on nutritional requirements for the identification of species was clearly shown by the many cases of "adaptation" of various strains to different substrates.

The experiments on the role of methionine certainly do not clarify the function of this compound in the metabolism of *P. phosphoreum*, and it seems likely that methionine may serve more than one purpose to the organisms. (For known functions, see Toennis, 1937; White, 1941; Lewis, 1941; also Harris and Kohn, 1941.) It seems clear that complex media are preferred by this species to those in which the minimum requirements are just satisfied, since the addition of a variety of non-essential substances serves not only to hasten development, but in some way to counteract the inhibitory action of compounds which are toxic when added singly. Cultures growing in the simplest media may, therefore, be thought of as artifacts; and the remarkable adaptability of the organisms, coupled with their sensitivity to external environmental factors leads to the tentative conclusion that each separate culture may be regarded as a delicately balanced system of living cells and their environment, each in a constant state of change and flux. As a further example of the changes in nutritional requirements of one of the strains of *P. phosphoreum* used in these studies, it need only be recalled that dissociates apparently unable to synthesize riboflavin were obtained in previous experiments, as well as "atavistic" back-dissociates from such deficient variants (Doudoroff 1938).

I wish to thank Professor C. L. A. Schmidt and Dr. H. Tarver of the Department of Biochemistry for their generous advice and gifts of certain amino acids used in these studios.

SUMMARY

1. *Photobacterium fischeri*, *P. splendidum*, *P. sepiae*, and *Achromobacter harveyi* were found to be capable of developing in inorganic media with simple organic compounds as the sole carbon source, while most strains of *Photobacterium phosphoreum* did not grow unless methionine was added as an accessory factor.

2. In experiments with one strain of *P. phosphoreum*, no compound or combi-

nation of compounds tried could replace methionine. Among the ten other strains tested, one was found for which no accessory factor whatsoever was essential, although methionine had a growth-promoting effect. Cultures of another strain studied could be "weaned" from methionine to accept homocystine as substitute, or even to develop in the absence of any accessory factor.

3. The complementary action of leucine and methionine, the "detoxification" of media to which certain inhibitory compounds were added by the addition of other compounds, and the stimulating effect of a variety of substances on the initiation of growth in certain media were observed in the studies with *P. phosphoreum*.

4. Seemingly anomalous effects of methionine and of mixtures of amino acids on growth, and the occurrence of "abortive" cultures were occasionally observed under certain conditions.

5. It would seem that a great many factors, including the previous history of the bacteria and the changes in both the organisms and their environment resulting from their growth play an important part in determining the course of development of each culture.

REFERENCES

Beijerinck, M W 1889 Le Photobacterium luminosum, bacterie lumineuse de la mer du nord Arch néerland sci, **23,** 401 405

1912 Mutation bei Mikroben Folia Microbiol, **1,** 1–97

1916 Die Leuchtbakterien der Nordsee im August und September. Folia Microbiol, **4,** 15 40

Doudoroff, M 1938 Lactoflavin and bacterial luminescence Enzymologia, **5,** 239–243.

1940 Experiments on the adaptation of Escherichia coli to sodium chloride J Gen Physiol, **5,** 585 611

Harris, J S and Kohn, H I 1941 On the mode of action of the sulfonamides II. The specific antagonism between methionine and the sulfonamides in Escherichia coli J Pharmacol, **73,** 383–400

Harvey, E N 1940 Living Light Princeton, N J, Princeton University Press

1941 Review of Bioluminescence Ann Rev Biochem, **10,** 531 552

Johnson, F. H 1938 Hexose oxidation by luminous bacteria III The escape from inhibition by alpha methylglucoside, with a note on urethanes J Cellular Comp Physiol, **12,** 281 294.

1939 Decomposition of hydrogen peroxide by catalase Nature, **144,** 634

Johnson, F H and Shunk, I V 1936 An interesting new species of luminous bacteria J. Bact, **31,** 585 592.

Lewis, H B. 1941 The significance of the sulfur-containing amino acids in metabolism. The Harvey Lectures, **36,** 159–187.

Pratje, A 1923 Das Leuchten der Organismen Munich verl. v. J F. Bergmann.

Toennis, G. 1937 The sulfur containing amino acid methionine Growth, **1,** 337 370.

White, A. 1941 The chemistry and metabolism of the compounds of sulfur Ann Rev. Biochem, **10,** 125-150.

STUDIES ON THE LUMINOUS BACTERIA

II. Some Observations on the Anaerobic Metabolism of Facultatively Anaerobic Species

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In view of the fact that all of the strains of luminous bacteria used in the present series of investigations (Doudoroff, 1942) were found to be capable of developing anaerobically in suitable media, it seemed desirable to study the fermentative dissimilations carried out by these organisms, in the hope of contributing to a better understanding of this group of bacteria from the physiological, taxonomic and ecological points of view. The problem seemed to be of particular interest because the luminous bacteria are morphologically related to a number of facultative anaerobes unquestionably belonging to the family Pseudomonadaceae, and at present scattered through the genera *Pseudomonas*, *Phytomonas*, *Flavobacterium*, and *Achromobacter*. The literature offers little information on the types of anaerobic metabolism to be found in such polarly flagellated organisms beyond the routine observations on acid and gas production with various carbohydrate substrates.

EXPERIMENTAL

Glucose was a satisfactory fermentable substrate for the growth of all the species studied in peptone-containing media under anaerobic conditions. The anaerobic development of *Photobacterium phosphoreum* and *Photobacterium fischeri* was poor in peptone media without sugar; *Photobacterium splendidum*, *Photobacterium sepiae*, and *Achromobacter harveyi* grew somewhat better, but still to a considerably lesser extent than with glucose. The addition of glycerol to peptone media did not increase the development of any of the species.

In glucose-peptone medium (consisting of M/30 Sörensen KH_2PO_4-Na_2HPO_4 buffer at pH 7.2 or 7.5 with 3% NaCl, 1% Bacto-Peptone and 0.5–1.0% glucose by weight) all strains produced acid, usually lowering the pH value to about 5.5, and only one species, *P. phosphoreum*, produced visible gas. Upon analysis of the products of fermentation, it appeared that except for minor differences, all of the species showed essentially the same type of sugar dissimilation, similar to the "mixed acid" fermentations characteristic of the Enterobacteriaceae. The principal products found were formic, acetic, lactic and succinic acids, ethyl alcohol, CO_2, and, in the case of *P. phosphoreum*, hydrogen.

Quantitative or semi-quantitative determinations of the products of fermentation were made with growing cultures of three strains of *P. phosphoreum* (Strains #2, #12, "Delft Strain") and with all of the other species under investigation. Sugar determinations were made with the ferricyanide reduction method de-

scribed by Hassid (1937). None of the major products of dissimilation were found to interfere with such determinations. Alcohol was distilled from the neutralized culture medium, redistilled with alkaline HgO (Friedman and Klaas, 1932) and quantitatively estimated by the reduction of acid dichromate. It was identified after oxidation to acetic acid (Duclaux distillation and sodium uranyl acetate). The organic acids were extracted from the culture medium after treatment with tungstic acid by liquid ether extraction and then separated into the steam-volatile and non-volatile fractions. Formic acid was identified microchemically as the cerous or lead salt and its amount computed from the weight of calomel formed by reduction of $HgCl_2$. Acetic acid was recognized as sodium uranyl acetate and estimated by subtraction of the amount of formic acid from the total volatile acid value. The correctness of the ratio so obtained was checked by Duclaux distillation of the volatile acid fraction. Lactic acid was identified microchemically as zinc lactate or by decomposition to acetaldehyde. Its quantity was determined by the method of Hartmann and Hillig (1933). Succinic acid was qualitatively and quantitatively determined by the use of succinic dehydrogenase preparations in a Warburg respirometer. Hydrogen was identified by quantitative combustion and both hydrogen and CO_2 were determined as free and dissolved gases with the help of the Van Slyke apparatus. Acetylmethylcarbinol was recognized through various modifications of the Voges-Proskauer test, carried out with distillates of the culture medium boiled with $FeCl_3$. 2,3-butylene glycol was identified and determined by the method of Kniphorst and Kruisheer (1937) with special precautions to avoid possible errors due to the presence of acetylmethylcarbinol.[1]

Fermentation of glucose by P. fischeri, P. splendidum, P. sepiae and A. hareyi

In glucose-peptone medium, the strains tested produced chiefly formic and acetic acids and ethyl alcohol. All produced some succinic acid as well as small amounts of CO_2. The principal difference observed among the various species appeared to be in the relative amounts of lactic acid formed. *P. fischeri* produced lactic acid in appreciable quantities, *P. harveyi* formed less, while *P. splendidum* and *P. sepiae* produced very little, if any, from sugar. Minute amounts of acetylmethylcarbinol could be detected in cultures of *P. fischeri* and *P. splendidum*, and slight traces with the other species, but no 2,3-butylene glycol was ever found among the products of fermentation, nor was hydrogen ever detected.

A fermentation balance obtained in an experiment with *P. fischeri* is presented in table 1. In the first column the figures represent the amounts of the various products of fermentation corrected only for blank determinations made with the uninoculated medium. In the second column, the quantities have been further corrected by subtraction of values obtained in a parallel experiment, in which the bacteria were allowed to grow in the peptone medium without added sugar.

[1] I wish to thank Mr. J. R. Gilliland for his kind cooperation in making the determinations of 2,3-butylene glycol, and in establishing the validity of the tests in the presence of the various constituents of the fermentation mixtures.

Carbon and "available hydrogen" balances based on the determinations (Barker, 1936) are included, as are the ratios of one-carbon to two-carbon derivatives found among the dissimilation products. In view of the generally accepted scheme for the formation of C_1 and C_2 compounds in sugar fermentation by the cleavage of three-carbon intermediates, the ratio of their derivatives serves as a valuable index to the validity of fermentation balances by comparison with the theoretical value of 1.0. Formic acid and CO_2 are the chief C_1 end products,

TABLE 1

Products of glucose dissimilation by P. fischeri and P. phosphoreum in glucose-peptone medium

Quantities of glucose utilized and of products recovered given in millimols, per 75 ml. of culture medium.

	P. FISCHERI*		P. PHOSPHOREUM†	
	A	B	A	B
Glucose utilized	0 850	0.850	0.694	0.694
Found:				
Hydrogen	0.00	0 00	0 380	0.380
CO_2	0 094	0.077	0.510	0.334
Formic acid	0.840	0.825	0.662	0.650
Acetic acid	0.602	0.484	0.428	0.304
Ethyl alcohol	0.540	0.540	0.560	0.560
Lactic acid	0 565	0.482	0.480	0.285
Succinic acid	0.084	0.062	0.062	0.042
Acetylmethylcarbinol	Trace	Trace	Trace	Trace
2,3-butylene glycol	0.000	0.000	0.004	0.004
Carbon recovered	103.3%	91.1%	113 6%	90.1%
Available hydrogen recovered	102.6%	91.4%	111.3%	89.7%
Ratio C_1/C_2 derivatives‡	0 82	0.88	1.24	1.18

Column A—amounts of fermentation products, corrected by subtraction of values obtained with uninoculated medium.

Column B—same, corrected by subtraction of values obtained with bacterial cultures in sugar-free medium.

* Medium initially at pH 7.5, containing 0.75% glucose.

† Medium initially at pH 7.2, containing 0.5% glucose.

‡ 2,3-butylene glycol included with 2-carbon compounds; succinic acid with one-carbon derivatives (see text).

while alcohol and acetic acid are the principal C_2 compounds. For the computation of C_1/C_2 ratio, succinic acid has been included with the C_1 derivatives in view of the accumulating evidence for the formation of succinic acid through the reduction of CO_2 and its addition to a three-carbon compound (Wood, C. W., 1941). That succinic acid may be produced in a similar manner by luminous bacteria is indicated by experimental findings with *P. phosphoreum* to be discussed further.

The small amounts of the products recovered make it difficult to place too much emphasis on the carbon and hydrogen balances, especially because of the large amount of peptone in the medium, which not only necessitated substantial corrections in some of the determinations, but made it impossible to conclude just how much of the products recovered had actually originated from the sugar. Small amounts of unidentified non-volatile acid were produced in media with, as well as without, sugar. From comparison of the values corrected for metabolites produced in sugar-free peptone medium (Column B) with those not so corrected (Column A) it would seem that the former give a slightly better agreement, but that the corrections are, most probably, justified only in part, or for some of the compounds rather than others. The good agreement of the carbon with the hydrogen balance may be more or less fortuitous, especially since unidentified products were certainly formed, and since the C_1/C_2 ratio was at best below 0.9.

In fermentations with *P. splendidum*, *P. sepiae* and *A. harveyi*, which grow to a somewhat greater extent without sugar, greater amounts of unidentified acidic products occurred, and it was even more difficult to decide how much of the recognized compounds arose from the dissimilation of sugar. Thus, the very small quantities of lactic acid produced by *P. sepiae* and *P. splendidum* in the presence of glucose were almost matched by the amounts formed in sugar-free peptone medium. Further experiments with resting cells, or with the use of synthetic media, may well be worth while for the elucidation of this problem.

Fermentations with P. phosphoreum

Fermentation of glucose. In addition to the compounds occurring in the anaerobic dissimilation of sugar by *P. fischeri*, hydrogen was produced by all the available strains of *P. phosphoreum*. It appeared only after the cultures were allowed to develop for some time and an appreciable amount of formic acid had accumulated. CO_2 appeared in relatively greater quantities than with *P. fischeri*. Some, though apparently not all, strains produced minute amounts of 2,3-butylene glycol; this compound seemed to appear in the later stages of fermentation, recalling the interesting observations by Mickelson and Werkman (1938), and Silverman and Werkman (1941) on the influence of the reaction of the culture medium on the production of acetylmethylcarbinol by *Aerobacter*.

Fermentation balances obtained with Strain #2 growing anaerobically in a glucose-peptone medium are presented in Table I. As with *P. fischeri*, subtraction of values obtained in cultures without glucose from the quantities of fermentation products recovered gives more reasonable balances, but the same uncertainty as to the validity of the corrections remains. The ratio of one-carbon to two-carbon derivatives is rather high, being in the neighborhood of 1.2. In the computation, 2,3-butylene glycol was treated as a C_2, and succinic acid as a C_1 derivative. Although the inclusion of succinic acid with the C_2 rather than with the C_1 derivatives would reduce the ratio to less than 1.1, this did not seem justified in view of the considerations set forth earlier and of experiments with resting cell suspensions to be reported further. It must be remembered that the dissimilation of the other constituents of the medium may be so altered by the

presence of sugar as to make the corrections applied to the analyses of the fermentation mixture too high or too low. Thus, if less acetic and more formic acid were produced from peptone in the presence than in the absence of glucose, the C_1/C_2 ratio would be materially reduced.

Some experiments were carried out with washed resting cells harvested from young cultures grown under semi-aerobic conditions in peptone-glycerol medium, the Warburg manometric technique being employed for the measurement of acid and gas production. Suspensions in phosphate buffers in a nitrogen atmosphere and in bicarbonate solutions with nitrogen and CO_2 in the gas phase were allowed to ferment known quantities of sugar to completion. Small amounts of CO_2 were produced as well as from $2\frac{1}{2}$ to $3\frac{1}{2}$ equivalents of acid per mol of glucose under such conditions. The CO_2 production was greatest in the early stages of the fermentation, decreasing or disappearing later. In fact, on several occasions, there was some evidence of a disappearance of minute amounts of CO_2 from the system in the later stages. No hydrogen production was observed in these experiments, possibly due to the small quantity of sugar decomposed.

Partial analysis of a sugar fermentation by washed resting cells in phosphate-free bicarbonate medium gave no evidence of the presence of lactic acid, but showed considerable amounts of succinic, as well as formic acids. This suggests that succinic acid may arise in place of lactic acid, possibly through the reduction of CO_2. (Wood *et al.* 1941). It was hoped that the addition of $CaCO_3$ to the medium in which cultures were allowed to develop would, in the same manner, favor the production of succinic acid, but an experiment to test this possibility failed to show any significant difference in the course of sugar dissimilation with and without $CaCO_3$, except for a slight inhibition of development and fermentation by the added chalk.

Decomposition of formic acid

Although it seems almost certain that formic hydrogenlyase is responsible for the formation of hydrogen and part of the CO_2, its presence was not demonstrated by the usual methods employing resting cell suspensions. This may have been due to the great difficulty of obtaining active cells. However, the addition of small amounts of formate to fermenting anaerobic cultures did lead to a corresponding increase in gas production.

Decomposition of pyruvic acid

Formic and acetic acids, CO_2, and hydrogen were identified as products of fermentation of pyruvic acid by growing cells of *P. phosphoreum*. As might be expected, no alcohol was found. Traces of acetylmethylcarbinol were detected, but no attempt was made to determine lactic and succinic acids.

Decomposition of alanine and fumaric acid

Since *P. phosphoreum* can develop to some extent anaerobically in peptone media without any added fermentable substance, and can utilize some amino acids (e.g., alanine) as chief carbon sources for aerobic growth, it appeared of in-

terest to test for the occurrence of the "Stickland reaction" with cultures of this species. Washed cell suspensions of Strain #2 were incubated anaerobically in Thunberg tubes in the presence of alanine together with some likely organic hydrogen acceptors, and qualitative tests for ammonia were made to determine whether a decomposition of alanine or other added amino acids had occurred. No ammonia was produced either from alanine alone or in mixtures of alanine with glycine, proline, pyruvate, or glucose. However, if alanine and furmarate were added together, a considerable evolution of ammonia resulted. It seems therefore, that although the classical "Stickland reaction" was not carried out under these conditions, alanine could be oxidized with fumaric acid acting as hydrogen acceptor, itself probably becoming reduced to succinic acid. Excellent anaerobic development could be obtained with fumarate, but not with succinate, added to peptone media. It seems probable that fumaric acid can be fermented (Barker, 1936) although no analyses were made with cultures so obtained. It is also possible that fumarate acts as an oxidizing agent for amino acids present in the peptone.

Autofermentation

The endogenous fermentation by resting cells could be shown manometrically to give rise to acidic products and small amounts of CO_2. Little if any ammonia was liberated, particularly if the cells were taken from young vigorous cultures. This may be taken as indirect evidence of the carbohydrate nature of the reserve products stored by the cells.

It is a well-known fact that many of the luminous bacteria produce acid when growing in a glycerol-containing medium under aerobic conditions. Aerobic development of *P. phosphoreum* in synthetic medium with glycerol as chief carbon source and methionine as nutrilite was accompanied by slight acid production and the presence of formic acid could be demonstrated in such cultures. Yet, no acid production could be detected in the respiration of glycerol by resting cells in a Warburg respirometer, and no fermentation of glycerol by cell suspensions or growing cultures was observed. No evidence for a "glycerohydrogenlyase" (Nakamura, 1940) was found. Whether the acid production is due to an autofermentation of reserve products in organisms which find themselves in the deoxygenated deep strata of the medium, or to an incomplete respiration of the glycerol to triose, followed by a fermentation of the latter, is impossible to decide at present.

I wish to express my sincere gratitude to Dr. C. B. van Niel of Stanford University and to Dr. H. A. Barker of the University of California for their kind advice and permission to use certain laboratory facilities and equipment.

SUMMARY AND CONCLUSIONS

1. All of the facultatively anaerobic species of luminous bacteria investigated showed essentially the same general "mixed acid" type of anaerobic sugar dissimilation.

2. Among the products formed were formic, acetic, lactic and succinic acids,

alcohol, CO_2, acetylmethylcarbinol, and, in fermentations with *Photobacterium phosphoreum*, hydrogen and occasionally 2,3-butylene glycol.

3. *Photobacterium fischeri*, *Photobacterium splendidum*, *Photobacterium sepiae*, and *Achromobacter harveyi* produced no hydrogen, and differed among themselves mainly in the extent of lactic acid production.

4. Some experiments with washed cell suspensions and growing cultures of *P. phosphoreum*, dealing with the anaerobic metabolism of glucose, formic, and pyruvic acids and alanine in the presence of fumaric acid are discussed.

REFERENCES

Barker, H. A. 1936 On the fermentation of some dibasic C_4-acids by *Aerobacter aerogenes*. Proc. Acad. Sci. Amsterdam, **39**, 674–683.

Doudoroff, M. 1942 Studies on the luminous bacteria. I Nutritional requirements of some species, with special reference to methionine. J. Bact , **44**, 451–459.

Friedemann, T. E., and Klaas R. 1936 The determination of ethyl alcohol. J. Biol. Chem., **115**, 47–61.

Hartmann, B. G., and Hillig, F. 1933 Determination of lactic acid in milk and milk products. J. Assoc Official Agr. Chem , **16**, 435–445.

Hassid, W. Z. 1937 Determination of sugars in plants. Ind. Eng. Chem., Anal. Ed., **9**, 228–229.

Kniphorst, L C. E., and Kruisheer, C. I. 1937 Die Bestimmung von 2,3-Butylenglycol, Acetylmethylcarbinol und Diabetyl in Wein und anderen Garungsprodukten. Z. Untersuch. Lebensm., **73**, 1–19.

Mickelson, M , and Werkman, C H. 1938. Influence of pH on the dissimilation of glucose by Aerobacter indologenes J Bact., **36**, 67–76.

Nakamura, H. 1940 Über die hydrogenlyase und die Hydrogenase in Leuchtbakterien, nebst einer Bemerkung uber die möglichkeit ihrer Beiteiligung an Leuchtvorgang. Acta Phytochim., **9**, 239–247

Silverman, M., and Werkman, C. H 1941 The formation of acetylmethylcarbinol from pyruvic acid by a bacterial enzyme preparation. J. Biol. Chem., **138**, 35–48

Wood, H. G., Werkman, C H., Hemingway, A., and Nier, A. O 1941 The position of carbon dioxide in succinic acid synthesized by heterotrophic bacteria. J. Biol. Chem., **139**, 377–381.

CULTURAL CHARACTERISTICS OF PENICILLIUM NOTATUM IN RELATION TO THE PRODUCTION OF ANTIBACTERIAL SUBSTANCE

Indication of the Dual Nature of the Antibacterial Substance[1]

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Of the bacterial antagonists thus far known to be produced by microorganisms, the most effective in bactericidal action against *Brucella abortus* is the culture filtrate from a strain of *Penicillium notatum* (Kocholaty, 1942). Another antibacterial substance, penicillin, also obtained from a culture of a strain of *Penicillium notatum*, but with very little potency against *Brucella abortus*, has been obtained and purified by a group of British workers (Abraham, Chain, Fletcher, Florey, Gardner, Heatley, and Jennings, 1941).

The work reported here deals chiefly with the cultural habits of a certain strain of *Penicillium notatum* with special regard to the production of an antibacterial substance against *Brucella abortus*. Distinction is drawn between penicillin and the antibacterial substance active against *Brucella abortus*, because the evidence thus far indicates that those two are not identical. As compared to previous results, it has been possible to increase the production of the antibacterial substance of the mold by about 10 times, so that 0.01 ml. of the crude culture filtrate, added to 10 ml. of tryptose agar will either strongly inhibit or completely suppress the growth of *Brucella abortus*. In addition to this the influence of different media, temperature, vitamins, heavy metal salts, etc., will be discussed.

Five different strains of *Penicillium notatum*, here called PEN 1, 2, 3, 4, 5, have been investigated; all strains with the exception of PEN 4[2] were obtained from Dr. S. A. Waksman's collection.[3]

Cultivation of those five strains on different solid media shows slight divergencies in growth, pigment and spore formation, etc.; on liquid media, all 5 strains show different production of antibacterial substances, PEN 2 being the most active in this respect, followed by PEN 6 and then PEN 4. Those three strains also showed distinctly the secretion of two different antibacterial substances. The other strains, producing less antibacterial substance, were not further investigated. Most of the experiments were carried out with PEN 2.

[1] This investigation is supported by the Thomas H. Dougherty, Jr. Fellowship in Research in Brucellosis Fund.

[2] Thanks are due to the Merck Company, Rahway, N. J., for this strain, which is a transfer of the strain the British workers were using in the preparation of penicillin.

[3] The writer is greatly indebted to Dr. S. A. Waksman for obtaining those strains, and also for the test organisms used in this work.

EXPERIMENTAL

Before discussing the influence of different factors upon growth and production of antibacterial substances, a few procedures, standardized for the sake of uniformity of results, are mentioned.

Inoculation. The mold is grown on glucose agar slants for 6 days at 28°. After this time spore formation is abundant. The spores are scraped off and suspended in about 8 ml. of saline, 1 ml. being used for the inoculation of a volume of about 70 ml. of medium, thus always insuring heavy growth, which results in a complete pellicle within 48 hours at 28°.

Incubation, growth, production of antibacterial substance. As compared with the temperature of 24°, used by the British workers, the incubation of PEN 2 was carried out at 28°, which in our experience gave the best results. Under those conditions an almost constant level of production of the antibacterial substance is reached around the fifth to sixth day at a pH of 3.5 to 4.0, using the modified Czapek-Dox medium, without addition of yeast extract or other vitamin source. After this time there are only slight changes in the activity of the filtrate. After about 10 days of incubation, at which a pH of about 7 is reached, the activity drops sharply and is practically at zero two days later; the pH at this time is approximately 8 or slightly greater. Pigment, spore formation, color of pellicle, etc., were found to be influenced by slight changes in the composition or sterilization of the medium and can therefore not be relied upon as characteristics. These features will be discussed in the respective sections.

Harvesting. At determined intervals, usually daily, the contents of 2 to 3 Erlenmeyer flasks were combined, in order to increase uniformity of result, and the antibacterial values of the crude culture filtrate were determined.

Medium. If not otherwise stated the medium was the modified Czapek-Dox medium, as suggested first by Clutterbuck, Lovell, and Raistrick, (1932). The depth of the medium was 17–20 mm.

The antibacterial test. In preference to the test in liquid medium or the assay method of the British workers, both of which are too awkward if many organisms have to be tested at short intervals, the streak test on solid media was preferred, as more economical in that it allows 4 organisms to be tested on the same plate. The culture filtrate of the mold, containing the antibacterial substance, diluted and adjusted if necessary to pH 5–6, was mixed with 10 ml. of tryptose agar. The test organisms from a 1- to 2-day culture were suspended in saline and streaked out on this medium. Readings were taken after 48 hours at 28° or 37°, depending on the organism tested. The growth was designated 0, 1, 2, 3; 0 meaning no growth, 3 full growth similar to control, and 1 and 2 intermediate stages of poor and fair growth.

Investigation of five different strains of Penicillium notatum for production of antibacterial substance against different test organisms. The five strains of *Penicillium notatum* were cultivated on the modified Czapek-Dox medium, sterilized under pressure. Each day changes in growth, mycelium, pH, production of

pigment, and antibacterial substance were noted. The antibacterial value of the culture fluid was assayed against 8 test organisms, 4 gram-positive, and 4 gram-negative, using the plate method as described.

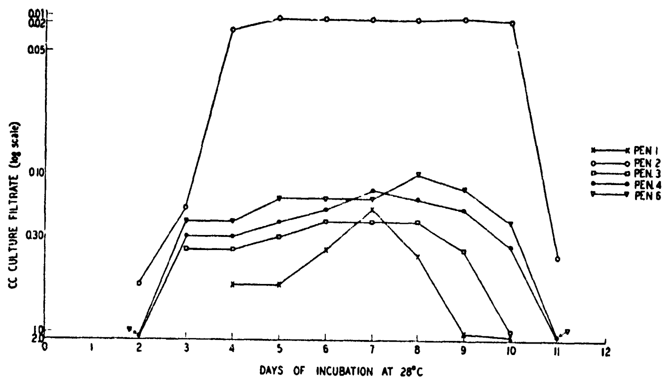

FIG. 1. SECRETION OF ANTIBACTERIAL SUBSTANCE, ACTIVE AGAINST BRUCELLA ABORTUS, BY FIVE DIFFERENT STRAINS OF PENICILLIUM NOTATUM

Abscissa: Days of incubation of five different strains of *Penicillium notatum*.

Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of *Brucella abortus* (streak test).

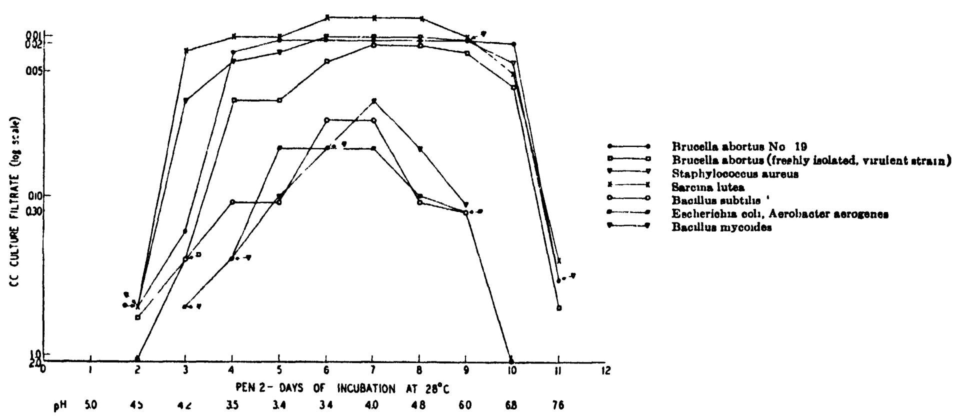

FIG. 2. PRODUCTION OF ANTIBACTERIAL SUBSTANCE DURING THE GROWTH OF PEN 2, AS ASSAYED AGAINST DIFFERENT TEST ORGANISMS

Abscissa: Days of incubation of PEN 2 at 28°; pH of the culture fluid day by day.

Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of the respective test organism, (streak test).

For obvious reasons not all the results can be published here, but figures 1 and 2 will suffice to demonstrate the effect. If one tabulates the results in such a way that all the values from total suppression to complete growth are expressed at correlate intervals, the "curves" give the minimal amount of crude culture fluid in ml. sufficient for complete suppression of the growth of the respective test organism in 10 ml. of tryptose agar (streak test). This way of expressing the

antibacterial activity of the filtrate will be retained for the other figures to follow. (For the sake of uniformity, fractions of ml. are spaced logarithmically.)

Although 8 different organisms were tested against each of the five strains of *Penicillium notatum*, day by day, figure 1 shows only the differences of the antibacterial substances as assayed against *Brucella abortus*, United States Bureau of Animal Industry, strain 19. Figure 2 gives a complete picture of the production of antibacterial substance by PEN 2 against 8 test organisms. Similar results, especially with regard to the relative sensitivity of those test organisms were obtained with the other 4 *Penicillium* strains, except that the absolute amount of antibacterial substance secreted by those strains was considerably less.

Since it was found that PEN 2 surpasses all other strains in the production of antibacterial substance, this strain was used for all the experiments reported here. It is worth mentioning that PEN 2 and PEN 6 both surpass PEN 4, (the strain used by the British workers for the purification of penicillin), in the production of antibacterial substance, not only against *Brucella abortus*, but also against *Staphylococcus aureus*. While all the rest of the strains of *Penicillium notatum* look quite similar to each other, especially on liquid medium, the pellicle of PEN 2 has an immediately recognizable fluffy appearance, an abundance of mycelium formation, strikingly different from the rest; the pellicle exceeds in thickness those of all other strains.

Influence of sterilization of the medium upon production of antibacterial substance. The modified Czapek-Dox medium was sterilized in three different ways: 1) 30 minutes under flowing steam for 3 consecutive days; 2) glucose was sterilized separately from the salt solution as a 56 per cent solution and added after sterilization in corresponding amounts to the salt solution; 3) the whole medium was sterilized under pressure.

The differences between sterilization 1), and 3) are practically negligible with regard to differences in the formation of antibacterial substance. Figure 2 represents the results of methods 1 and 3. If glucose is sterilized separately according to method 2), differences are evident, as shown in figure 3.

Apart from minor changes—shifting of the peak of the production of the antibacterial substance for instance—the formation and disappearance of an antagonistic substance acting against *Escherichia coli* and *Aerobacter aerogenes*—but not acting against *Brucella abortus*—is most striking. While on the fifth day of growth an amount of 0.05 to 0.1 ml. of the culture fluid will suppress the growth of *Escherichia coli*, *Aerobacter aerogenes*, *Staphylococcus aureus* or *Brucella abortus*, 4 days later even 100–200 times the amount capable of suppressing the growth of *Brucella abortus* or *Staphylococcus aureus* is insufficient to cause even a slight inhibition of *Escherichia coli* or *Aerobacter aerogenes*. This fact is only to be explained by the formation of two different antibacterial substances, influenced by the detail of sterilizing the glucose separate from the salts.

Apart from this, other changes are apparent. The pigment production of the medium sterilized under pressure or especially with flowing steam is rather meager, and in color a light yellow-green, becoming darker only in the latest stages of growth, in which the antibacterial substance disappears; in contrast

the pigment production after sterilizing glucose separately is on the fourth day a faint orange, becoming almost brown at the peak of the production of antibacterial substance. The under side of the mycelium if the medium is sterilized with flowing steam or under pressure is first white, in the latter stages brownish; if sterilized according to method 2) it is bright yellow-green.

This difference in the production and activity of antibacterial substance against *Escherichia coli* and *Aerobacter aerogenes* was also possible to demonstrate on strains PEN 4 and PEN 6 to a somewhat lesser extent; the remainder of the strains showed too little antibacterial activity to make such changes detectable.

Influence of heavy metal salts. Substituting for the 10 mg of $FeSO_4 \cdot 7H_2O$ per liter in the modified Czapek-Dox medium equivalent amounts of the sulfates of Zn, Cu, and Mn, the results obtained are represented in figure 4 against *Brucella abortus* as test organism. Also tested were *Staphylococcus aureus*,

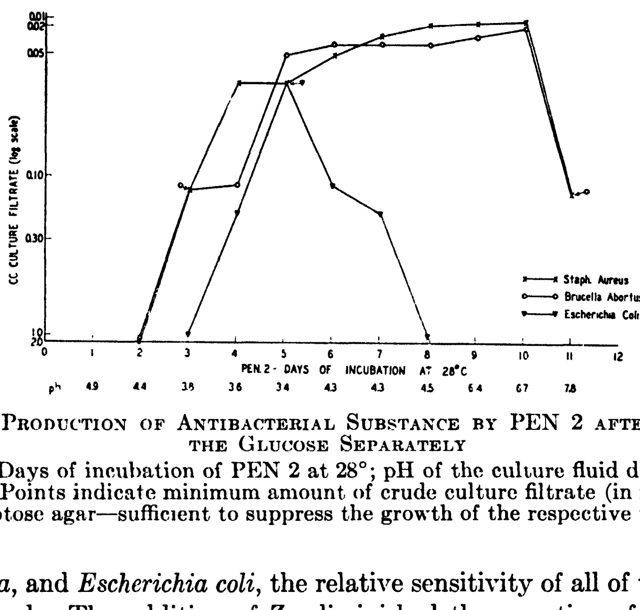

FIG. 3. THE PRODUCTION OF ANTIBACTERIAL SUBSTANCE BY PEN 2 AFTER STERILIZING THE GLUCOSE SEPARATELY

Abscissa: Days of incubation of PEN 2 at 28°; pH of the culture fluid day by day.
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of the respective test organisms, (streak test).

Sarcina lutea, and *Escherichia coli*, the relative sensitivity of all of them remaining unchanged. The addition of Zn diminished the secretion of antibacterial substance as compared with Fe, but the growth of the mold was superior in its development and heaviness of pellicle as compared with Fe. In contrast to this, the addition of Cu resulted in a very meager growth, resembling in its appearance very much the growth which is obtained when the mold is grown on a slant at 37°. No regular pellicle was formed, only spots of mycelium on the surface and rim of the vessel were obtained, presenting a "crowded" white growth of brittle mycelium at best covering only 10 to 20 per cent of the available surface area. In relation to this meager growth the production of antibacterial substance seems rather high.

The influence of Mn ions is outstanding; Mn not only replaces but surpasses Fe in increasing heaviness of growth and production of antibacterial substance. At the peak of the secretion of antibacterial substance, 0.005 ml. of the crude

culture filtrate was able to suppress the growth of *Brucella abortus* or *Staphylococcus aureus* and 0.001 ml. was sufficient to suppress the growth of *Sarcina lutea* (the most sensitive test organism used), in 10 ml. of tryptose agar.

Temperature, growth and production of antibacterial substance. Clutterbuck, Lovell, and Raistrick (1932) grew the mold at 25°, the other British workers (1941) at 24°, while here the temperature of 28° was used exclusively. The finding of Fleming (1929) that the mold will grow at 37° was confirmed (with PEN 2); the mold will cover within 14 days a Czapek-Dox agar slant, (very much less growth is obtained on glucose agar slant), with a crowded brittle growth similar to that obtained by substitution of Cu for Fe in the liquid medium at 28°. Slow growth is also obtained at refrigerator temperature, a Czapek-Dox slant being covered within 14 days with a fluffy white growth. If a flask of a mold

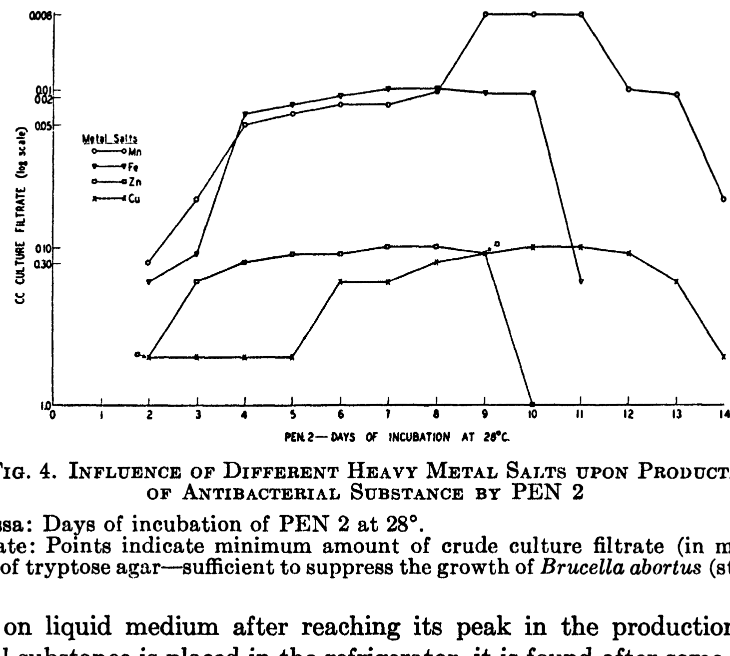

FIG. 4. INFLUENCE OF DIFFERENT HEAVY METAL SALTS UPON PRODUCTION OF ANTIBACTERIAL SUBSTANCE BY PEN 2

Abscissa: Days of incubation of PEN 2 at 28°.
Ordinate: Points indicate minimum amount of crude culture filtrate (in ml.)—added to 10 ml. of tryptose agar—sufficient to suppress the growth of *Brucella abortus* (streak test).

culture on liquid medium after reaching its peak in the production of antibacterial substance is placed in the refrigerator, it is found after some days that the activity of the antibacterial substance is increased. Whether this increase is due to some slight additional growth, or to the diffusion of active material from the pellicle into the culture fluid was not investigated.

Certain signs, however, speak for the possibility that conditions optimal for growth are not necessarily optimal for production of penicillin; (see for instance the influence of Zn, Cu). In one instance the mold was grown at room temperature, (about 20°). Although the pellicle formed was thin and the growth retarded for a longer period of time, the amount of production of antibacterial substance was about the same as if the mold were incubated at 28°. In another case the addition of $CaCO_3$ was tried and although the pellicle obtained after 14 days of growth at 28° was almost paper-thin, with no pigment formation at

all, the production of antibacterial substance again was normal. All these findings seem to indicate that optimal growth, or optimal temperature for growth, or optimal pellicle formation, are no indication for optimal production of antibacterial substance.

If grown at 28° on modified Czapek-Dox agar the mold (PEN 2) forms a complete pellicle within 2 days, at which time the formation of antibacterial substance can be detected in the culture fluid. The changes in pH during the successive stages of growth, at first an increase toward the acid side, later a slow decrease in acidity, and finally a rapid rise in the pH are about the same as reported by other workers. (See also figures 1 and 2.)

Composition of medium. In tryptone medium with the necessary salts, with or without glucose, the pH rises after 6 days to about 8, and production of antibacterial substance is not demonstrable. Substituting ammonium sulfate for $NaNO_3$ as nitrogen source in the Czapek-Dox medium lowers the production of antibacterial substance. However, doubling the nitrogen and carbon source results in some increase in the production of antibacterial substance, resembling very much the results obtained when Fe in the medium is replaced with Mn.[4]

From the few experiments conducted regarding the utilization of glucose, it was found consistently, whether 100 g or 40 g of glucose were used per liter of Czapek-Dox medium, that this source of carbon was rapidly utilized, 90 per cent or more of the glucose being used up at the optimum of the production of antibacterial substance. Determination of acidity carried out at the same time showed no characteristics worth mentioning.

It has been reported by different investigators (Abraham, Chain, Fletcher, Florey, Gardner, Heatley, and Jennings, 1941; Clutterbuck, Lovell, and Raistrick, 1932) that occasionally batches of the culture filtrate of the mold are obtained, apparently free of contamination, without any antibacterial activity. The strain of PEN 2 used for almost all of the experiments reported in this paper for more than 8 months has not given a single batch of material without antibacterial activity, parallel determinations showing great uniformity.

DISCUSSION

Except for the experiments recorded in figure 2, where 8 test organisms were used, there were used 4 test organisms, namely *Brucella abortus*, *Staphylococcus aureus*, *Escherichia coli*, and *Sarcina lutea*. The peak of the production of antibacterial substance always showed the following relationship in sensitivity of these four organisms: *Staphylococcus aureus*:*Brucella abortus*:*Escherichia coli* = 1:1–2:10, i.e., if one ml. of the culture filtrate will suppress *Staphylococcus aureus*, 1–2 ml. are necessary to suppress growth of *Brucella abortus* and 10 ml. *Escherichia coli*. (*Sarcina lutea* is much more sensitive than *Staphylococcus aureus*, its relation being about 0.3.) This relation between these four organisms remained unaltered in all the experiments.

The only evidence brought forth in this paper that two different substances

[4] Thiamin hydrochloride, pyridoxin, or riboflavin, added to the culture fluid were without influence upon either growth of the mold or secretion of antibacterial substance.

are secreted by the mold (recorded in figure 3) is afforded by the substance acting against *Escherichia coli.* If one compares the crude culture filtrate of the mold as found here, however, with the more than 1000 times purified penicillin of the British workers (1941), another difference is apparent. The sensitivity of *Staphylococcus aureus*:*Brucella abortus*:*Escherichia coli* against the highly purified penicillin compares as 1:500:1000, as against the ratio with the crude culture filtrate of PEN 2 of 1:1–2:10. Even assuming slight variations in the sensitivity of the test organisms, these would not suffice to reconcile those discrepancies.

In the purification of the penicillin, the British workers (1941) probably used *Staphylococcus aureus* as a test organism. It might well be conceivable that in using *Escherichia coli*, for instance, a substance with entirely different antibacterial properties against those three organisms might have been isolated. Furthermore, the possibility that two different antibacterial substances might be formed by the mold would fit into the picture of other soil organisms producing two antibacterial substances such as the Gramicidin and Tyrocidine produced by *Bacillus brevis*, (Dubos, 1939), and Actinomycin A and B produced by *Actinomyces antibioticus*, (Waksman, 1941). Regarding the two substances secreted by the mold it must be said that these two substances need not necessarily be quite different. It could be that only slight changes in one and the same substance—for instance during the growth of the mold—might produce the difference in their antibacterial properties. Another possibility is that in the purification of penicillin the original substance might have been slightly altered in its original antibacterial properties. Only the purification of the antibacterial material can bring the desired evidence.

SUMMARY

1. Several strains of *Penicillium notatum* investigated were found to vary widely in their property of producing penicillin.

2. The optimal growth of the mold, or optimal temperature for the growth of the mold does not necessarily coincide with the optimal production of antibacterial substance.

3. The mode in which the (glucose-containing) medium is sterilized shows divergencies in the production of antibacterial substances as well as other differences.

4. The influence of other heavy metal salts than Fe was studied and Mn was found to surpass Fe in production of antibacterial substance.

5. Among five strains of *Penicillium notatum* tested for the production of antibacterial substances, it was found that at least three strains—all varying widely in their production of antibacterial substance,—seem to produce 2 different substances which differ in their antibacterial properties. Using optimal conditions, so far as they are known as yet, a strain of *Penicillium notatum* was found to produce an antibacterial substance, which in amounts of 0.01 ml. of the crude culture filtrate added to 10 ml. of tryptose agar, will prevent the growth of *Brucella abortus*.

6. The difference between purified penicillin and the substance found in the crude culture filtrate is discussed with reference to the possibility of the dual nature of the antibacterial material produced by the mold.

REFERENCES

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A. 1941 Further observations on penicillin. Lancet, **2**, 177–189.

Clutterbuck, P. W., Lovell, R., and Raistrick, H. 1932 The formation from glucose by member of the *Penicillium chrysogenum* series of a pigment, an alkali soluble protein and penicillin—the antibacterial substance of Fleming. Biochem. J., **26**, 1907–1921.

Dubos, R. J. 1939 Bactericidal effect of an extract of a soil bacillus on gram-positive cocci. J. Exptl. Med , **70**, 1–17, 249–256.

Fleming, A. 1929 On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. Brit. J. Exptl. Path., **10**, 226–236.

Kocholaty, W. 1942 Investigations in micro-organisms antagonistic to *Brucella abortus*. In preparation.

Waksman, S. A., and Woodruff, H. B. 1941 Antinomyces antibioticus a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. J. Bact., **42**, 231–249.

PYRIDOXINE NUTRITION OF LACTIC ACID BACTERIA[1]

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Previous reports from this laboratory have shown that pantothenic acid is essential for the growth of every species of lactic acid bacteria tested (Snell *et al.*, 1937). It has also been shown that while some species of lactic acid bacteria require riboflavin for growth, several do not (Snell and Strong, 1939). In a study of the activity of certain synthetic flavins, a close correlation was found to exist between the rat assay and the bacterial test (Snell and Strong, 1939). Vitamin B_6 (pyridoxine) has been shown to be necessary for the growth of certain lactic acid bacteria (Möller, 1938). Möller has shown that 2:4-dimethyl-3-hydroxy-5-hydroxymethyl pyridine has a very small vitamin B_6 activity and that 2,4,5-trimethyl-3-hydroxypyridine has no activity when tested with *Streptobacterium plantarum* 10S (Möller *et al.*, 1939).

The objectives of this present study were, first, to examine the vitamin B_6 requirements of several species of lactic acid bacteria and, second, to determine the activity of some vitamin B_6 analogues for *Lactobacillus casei*[2] with the intention of comparing the observed results with the reported antidermatitic activity of these compounds on rats.

CULTURE MEDIUM

The basal medium used was such that maximum acidity could be produced by the organism, *Lactobacillus casei*, when sufficient pyridoxine was added. The composition of the medium was as follows:

Acid-hydrolyzed alcohol-extracted casein	0.5 per cent
Glucose	1.0 per cent
Sodium acetate	0.6 per cent
Tryptophane	0.01 per cent
Cystine	0.01 per cent
Riboflavin	0.01 mg. per cent
Nicotinic acid	0.02 mg. per cent
Pantothenic acid	0.02 mg. per cent
Acid ether extract of Galen B	5.0 mg. per cent
Alcohol-extracted solubilized liver	2.0 mg. per cent

Inorganic salts (K_2HPO_4 0.5 gm.; KH_2PO_4 0.5 gm.; $MgSO_4 \cdot 7H_2O$ 0.2 gm.; NaCl 0.01 gm.; $FeSO_4 \cdot 7H_2O$ 0.01 gm.; $MnSO_4 \cdot 3H_2O$ 0.01 gm.; in 1000 ml. of medium.)

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[2] This is the organism which has been widely used for the determination of riboflavin and pantothenic acid and recently adapted for biotin assay. It has usually been called *L. casei* ε but also has been designated as *L. casei* and *L. helveticus*. In accordance with the views of several bacteriologists who have been consulted and in accordance with the report of Tittsler *et al.* (1942), the name *Lactobacillus casei* American Type Culture Collection No. 7469 is used in this paper.

To free the casein of vitamin B_6 activity it was given the following treatment. One hundred grams of Labco casein were ground to pass through a No. 80 mesh sieve and then refluxed 3 times, 3 hours each time, twice with 350 ml. of absolute alcohol and finally with 350 ml. of 95 per cent ethanol. The casein was then dissolved in 3 liters of dilute ammonium hydroxide and precipitated with hydrochloric acid, dried and ground to pass through a No. 100 mesh sieve and refluxed once more for 3 hours with 350 ml. of absolute ethanol and once more for 3 hours with 350 ml. of 95 per cent ethanol. The yield was around 60 grams. To prepare the casein hydrolysates 20 grams of the alcohol-extracted casein were autoclaved at 20 lbs. pressure with 800 ml. of 10 per cent HCl for 3 hours. The HCl was removed *in vacuo*, the solution was adjusted to pH 6.8 and then diluted to make a 5 per cent concentration of hydrolyzed casein.

The acid ether extract of Galen B[3] was made by extracting a 20 per cent solution of Galen B at pH 2.5 with peroxide-free ether in a Kutcher-Steudel extractor for 72 hours, changing the ether every 24 hours. The ether extract was concentrated to dryness and then made up to a concentration of 5 mg. per ml.

Five grams of solubilized liver[4] were refluxed with 100 ml. of absolute ethyl alcohol for 3 hours and then filtered. The extraction was repeated 3 times. The extracted solubilized liver was dried and dissolved as needed.

CULTURES

The cultures were as follows: *Lactobacillus arabinosus* 17-5; *Lactobacillus casei; Lactobacillus delbrückii* 3; *Lactobacillus pentosus* 124-2; *Lactobacillus lactis* Bl-1; and *Leuconostoc mesenteroides* P-60. The organisms were carried as stab cultures in yeast water glucose agar.

TECHNIQUE OF BIOLOGICAL TESTING

The inocula were prepared as follows: The organisms were grown in 0.5 per cent peptone, 0.6 per cent sodium acetate, 1 per cent glucose, inorganic salts medium. Transfers were made every 24 hours. For inocula, second or third transfers were used. The cells in 10 ml. of a 24-hour culture were centrifuged down, suspended in 10 ml. sterile 0.9 per cent saline solution, and 0.1 ml. of this suspension was added to 80 ml. of sterile 0.9 per cent saline solution and thoroughly mixed. 0.3 ml. of this last suspension was used as inoculum for each tube. This inoculum was used in all experiments unless otherwise indicated.

The growth tests were carried out in test tubes containing 10 ml. of medium at pH 6.8. The basal medium was first made up at 1.25 times the above concentration (8 ml. per tube) and the test material was incorporated in a 2 ml.

[3] We are indebted to the Galen Co., Inc., Berkeley, California for the gift of Galen B.

[4] The solubilized liver fraction is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water soluble by enzyme action. We wish to thank Dr. David Klein, of the Wilson Laboratories, Chicago, for this preparation.

addition. The tubes were autoclaved at 15 lbs. pressure for 15 minutes, cooled and inoculated.

Cultures of *L. mesenteroides*, *L. arabinosus* and *L. pentosus* were incubated at 25°C., the other three bacteria were incubated at 37°C. Growth observations were made by turbidity readings with the Evelyn photoelectric colorimeter. Fermentation was followed by titration of acid produced after 3 days growth.

In a comparison of the various tables it will be found that *L. casei* does not give a standard response to a given level of pyridoxine. It has been impossible to control this variation to any great extent. However, it is known to be associated with age of culture, number of subcultures from the stock culture and the amount of pyridoxine present in the culture medium. Data for each table are taken from the same experiment. The individual experiments were repeated a number of times and gave the same relative values but the absolute values differed.

TABLE 1

Effect of size of inoculum on requirement for pyridoxine

INOCULUM	MICROGRAMS OF PYRIDOXINE PER 10 ML OF MEDIUM				
	0	0.2	0.4	1.0	2.0
	ml N/10 acid	*ml N/10 acid*	*ml. N/10 acid*	*ml. N/10 acid*	*ml. N/10 acid*
A	3.5	4.6	10.2	10.6	10.2
	6.2	4.8	8.6	10.1	10.2
B	1.0	5.6	6.3	10.4	10.2
	1.0	3.5	5.4	10.2	10.4
C	0.4	0.8	2.8	9.5	10.1
	0.5	1.0	2.6	9.5	10.3

PYRIDOXINE REQUIREMENTS OF L. CASEI

The amount of pyridoxine necessary for maximum growth and acid production depends on the size of the inoculum. This was readily demonstrated by the following experiment. The cells from 10 ml. of a 24-hour culture grown in the peptone medium were centrifuged, suspended in 10 ml. of sterile saline solution, centrifuged again and resuspended in 10 ml. of saline. Inocula of three concentrations were prepared as follows: Inoculum A was prepared by diluting 0.5 ml. of the stock suspension with 10 ml. of saline, inoculum B by diluting 0.5 ml. of A with 10 ml. of saline, and inoculum C by diluting 0.5 ml. of B with 10 ml. of saline. 0.1 ml. of the respective dilutions was used for each tube. The data are summarized in table 1.

That these effects were not due to mechanical carry over with the cells was shown by additional washing of the cells with saline solution. The extra washing did not alter the results.

The effect noted in table 1 is probably due to the storage of more pyridoxine

by the cell than is necessary for its growth and function. The ability of *L. casei* to store an amount of a specific factor above the physiological requirements of the cell has also been observed in respect to biotin and the norit eluate factor. No such storage of riboflavin or pantothenic acid has been observed.

It is evident that, if the organism is to be used as an assay agent, it must be grown on a sub-optimum, if not a minimum, amount of the vitamin. This would prevent storage of the growth factor but allow the organism a sufficient amount of the factor for the life processes of the cell.

The effect of various methods of oxygen removal was studied. Some tubes were incubated in anaerobic jars, to some metallic iron was added, and to others 0.3 mg. $NaHSO_2$. It was found that the vitamin requirements were increased, in the order of the methods mentioned (table 2). The effect of $NaHSO_2$ seems to be due to oxygen removal rather than to toxicity of the compound. This is probable since at the higher levels of pyridoxine the organism

TABLE 2

Effect of oxygen removal on the pyridoxine requirement of L. casei

PYRIDOXINE ADDED	TUBES INCUBATED IN AIR	TUBES INCUBATED IN ANAEROBIC JARS	TUBES CONTAINING REDUCED IRON	TUBES CONTAINING 0 3 MG. $NaHSO_2$ PER 10 ML.
µg./10 ml.	*ml. N/10 acid/10 ml.*	*ml. N/10 acid/10 ml.*	*ml N/10 acid/10 ml.*	*ml. N/10 acid/10 ml.*
0.1	6.8	5 6		0.8
0.2	9 0	6.2		0.8
0.4	8 6	7.2	0 2	0.8
0 8	8.9	8 4	0.25	2.2
1 2	9 9	8.7	4.7	2.0
1.6	10.1	9.0	7.1	2 0
2 0	10.0	9.7	8 5	5 0

is producing significant amounts of acid. It is unlikely that pyridoxine would function as a specific antagonist for $NaHSO_2$ if this compound were toxic.

The role that vitamin B_6 plays in cellular metabolism is not known, but it is significant that the bacterial cell requires more of the vitamin when the oxygen tension is reduced.

PYRIDOXINE REQUIREMENTS OF VARIOUS SPECIES OF LACTIC ACID BACTERIA

Requirement studies were made with tubes containing the basal medium, and tubes with 0.2 microgram of pyridoxine added per ml. of the basal medium. Each succeeding subculture was inoculated with cells taken from the preceding tube in the series. The results are shown in table 3.

It is obvious that *L. casei*, *L. delbrückii*, and *L. lactis* require pyridoxine in the media. *L. arabinosus* and *L. pentosus* grow just as well without pyridoxine as with it but the growth of *L. mesenteroides* is stimulated by addition of the vitamin.

To show the synthesis of vitamin B_6 by bacteria which grew in the basal medium the cells were centrifuged out, suspended in 10 ml. of water and auto-

lyzed under toluene. The centrifuged cell-free media were neutralized and concentrated to 10 ml. volumes. The cell autolysates and culture filtrates were tested for vitamin B_6 activity with *L. casei* as the assay organism. The ml. of acid produced furnish an index of the vitamin B_6 present (table 4). It was found that those bacteria requiring added pyridoxine did not synthesize the vitamin, whereas the others did. *L. pentosus* and *L. arabinosus* synthesized approximately the same amount of pyridoxine. *L. mesenteroides* synthesized about one-fourth as much pyridoxine as did the other organisms. This limited synthesis by *L. mesenteroides* is in accord with the earlier observation (table 3)

TABLE 3

Growth of successive subcultures of lactic acid bacteria in the presence and absence of pyridoxine

(Figures are readings on Evelyn colorimeter)

ORGANISM	NO PYRIDOXINE			0.2 MICROGRAMS PYRIDOXINE PER ML.		
	First	Second	Third	First	Second	Third
L. arabinosus 17-5	31	33	29	29	30	30
L. casei	89	91	91	26	26	30
L. delbrückii 3	88	88	88	57	60	61
L. pentosus 124-2	39	40	39	37	42	40
L. lactis Bl-1	86	79	71	49	56	53
L. mesenteroides P-60	62	62	60	55	59	54

TABLE 4

*Pyridoxine synthesis by certain lactic acid bacteria**

ORGANISM	AGE OF CULTURE	ML. OF ADDED FILTRATE OR AUTOLYZED CELL SUSPENSION					
		0 5		1.0		2 0	
		Filtrate	Cell	Filtrate	Cell	Filtrate	Cell
	hours						
L. lactis Bl-1	72	0.6	0.6	0.6	0.6	0 6	0.8
L. mesenteroides P-60	72	0.6	0.6	0.6	0.6	2 1	1 3
L. pentosus 124-2	72	2.4	0 8	7.5	1.8	10.4	4.5
L. arabinosus 17-5	72	2.4	1.5	5.7	1.9	10.4	4.7

* The figures are the ml. of $N/10$ acid produced per 10 ml. of medium in the assay culture.

that the growth of *L. mesenteroides* is stimulated by addition of the vitamin. Under the conditions of the test *L. mesenteroides* cannot synthesize pyridoxine at a rate sufficient to meet the requirements of the cell while *L. pentosus* and *L. arabinosus* can.

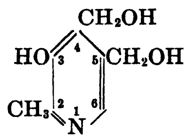
From the above data it is possible to calculate the percentage of pyridoxine present in the cell and the percentage present in the medium. In all cases the largest amount of the vitamin was present outside of the cell. This may be due to the secretion of pyridoxine into the medium or it may be due to autolysis of the cell and consequent liberation of the vitamin.

EFFECT OF PYRIDOXINE ANALOGUES ON THE GROWTH OF L. CASEI

The basal medium was supplemented with varying amounts of each of the analogues[5] and the acidity produced was compared with that when pyridoxine was added. Autoclaving solutions of compounds II and III results in hydrolysis to pyridoxine. Therefore solutions of these compounds were sterilized by filtering through a Seitz filter and adding the filtrate to the autoclaved basal medium. The remainder of the compounds were incorporated in the basal medium and autoclaved at 15 lbs. pressure for 15 minutes. The inactive compounds were tested at concentrations up to 2 micrograms per ml. The results obtained with the different pyridine derivatives are recorded in table 5.

TABLE 5

Activity of pyridoxine (vitamin B_6) derivatives

COMPOUND	ACTIVITY*
	
2-Methyl-3-hydroxy-4,5-bis(hydroxymethyl) pyridine (pyridoxine)	1.0
Derivatives of 2-methyl pyridine	
I. 3-Hydroxy-4,5-bis-(acetoxymethyl)-	0.8–1.0
II. 3-Acetoxy-4,5-bis-(acetoxymethyl)-	0
III. 3-Hydroxy-4,5-bis-(bromomethyl)-	0.6–0.8
IV. 3-Amino-4-bromomethyl-5-aminomethyl-	0
V. 3-Amino-4-hydroxymethyl-5-aminomethyl-	0
VI. 3-Amino-4-ethoxymethyl-5-aminomethyl-	0
VII. 3-Hydroxy-4-ethoxymethyl-5-hydroxymethyl-	0 3
VIII. 3-Hydroxy-4-methoxymethyl-5-hydroxymethyl-	0.3–0.4
IX. 3-Hydroxy-4,5-epoxydimethyl-	0 2–0.3
X. 3-Hydroxy-4-methyl-5-hydroxymethyl-	0.03 (?)
XI. 3-Hydroxy-4,5-dimethyl-	0
XII. Lactone of 3-hydroxy-4-hydroxymethyl-5-carboxy-	0
XIII. Lactone of 3-amino-4-hydroxymethyl-5-carboxy-	0

* Activity of on molar basis.

No analogues of pyridoxine were found to be as active as the pure vitamin. The diacetyl compound (I) was found to be nearly as active as the vitamin but the triacetyl compound (II) was inactive. In the rat assay (Unna, 1940), the di- and tri-acetates were found equally potent and of the same activity as the vitamin. Apparently the bacteria could not hydrolyze the acetate in the 3 position whereas the rat could bring about this hydrolysis. Replacement of the hydroxyls of the hydroxymethyl groups by bromine reduced the activity about 40 per cent. It was not determined whether or not the activity was due

[5] We are indebted to Dr. Karl Folkers of Merck and Company, Inc., Rahway, New Jersey, for the gift of these compounds.

to the hydrolysis of the bromines, but it was found that the solution resulting from autoclaving the compound had the same activity as the vitamin when equimolecular amounts were tested.

Replacement of the phenolic hydroxyl and the hydroxyl of the 5-hydroxymethyl group by amino groups resulted in a compound which was inactive (V), as were the derivatives of this compound (IV, VI). It may be that the lack of activity was due to the replacement of the hydroxyl in the 3 position.

When the 4-hydroxymethyl group was methylated (VIII) or ethylated (VII) there was a loss of about 70 per cent of activity. Between the two ethers a slight difference in potency was observed. A further reduction in activity resulted from the formation of an inner ether between the two hydroxyl groups in the 4 and 5 positions (IX). It is highly improbable that the organism could hydrolyze the ether linkages in these compounds. Rat assay showed much the same activity of these ethers but lesser activity of the inner ether.

When one hydroxymethyl group was replaced by a methyl group, the compound (X) was found to have very little activity, but when both the hydroxymethyl groups were replaced by methyl groups (XI) no activity was found. These results agree with those of Möller. Both of these compounds were inactive when tested with rats.

The lactone of the compound, formed by substituting a carboxyl for a hydroxymethyl group (XII), was found to be inactive for the bacteria, just as it had been found inactive for rats.

SUMMARY

Lactobacillus casei has been shown to store pyridoxine in amounts greater than are necessary for growth. The pyridoxine requirement was dependent on the oxygen tension of the medium.

Three of the six species of lactic acid bacteria investigated do not require pyridoxine for growth and acid production and they were all shown to synthesize the vitamin when cultured on a medium devoid of pyridoxine.

The response of *L. casei* to a number of pyridoxine analogues parallels rather closely the antidermatitic effect of these same compounds on rats.

REFERENCES

Moller, E. F. 1938 Vitamin B_6 (Adermin) als Wuchstoff fur Milchsaurebakterien. Z. physiol. chem., **254**: 285–286.

Moller, E. F., Zima, O., Jung, F., and Moll, T. 1939 Biological comparison of synthetic and natural adermin. Naturwissenschaften **27**: 228–229.

Snell, E. E., Strong, F. M., and Peterson, W. H. 1937 Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. Biochem. J., **31**: 1789–1799.

Snell, E. E., and Strong, F. M. 1939 The effect of riboflavin and certain synthetic flavins on the growth of lactic acid bacteria. Enzymologia **6**: 186–193.

Tittsler, Ralph P., Rogosa, Morrison, and Whittier, Earle O. 1942 The nomenclature and characteristics of the lactobacillus employed for the microbiological assay of riboflavin. J. Bact., **43**: 56.

Unna, Klaus. 1940 Antidermatitic effect of vitamin B_6 analogues. Proc. Soc. Exptl. Biol. Med., **43**: 122–124.

DIFFERENTIATION OF THE "INTERMEDIATE" COLI-LIKE BACTERIA

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I. INTRODUCTION

"Intermediate" coli-like bacteria have received much attention since Koser (1923) first reported that *Escherichia coli* did not produce vigorous growth when citrate was present as a sole source of carbon and later (1924) found cultures, similar to *Escherichia coli* in other characteristics, which grew as luxuriantly in the citrate medium as did the *Aerobacter* cultures.

Study of these citrate-utilizing, coli-like bacteria was first confined to confirmation of Koser's work; general recognition of their wide distribution in nature and study of other characters which would differentiate these "Intermediate" forms (Methyl-red positive; Voges-Proskauer negative; citrate positive) from true *Escherichia* (M.R.+; V.-P.−; citrate−) and *Aerobacter* (M.R.−; V.-P.+; citrate+) soon followed.

Bardsley (1926), Raghavachari (1926), Taylor *et al.* (1927), Hill *et al.* (1929), Lewis and Pittman (1928), Holwerda (1928, 1930), Minkewitsch *et al.* (1928), Minkewitsch (1930), Brown and Skinner (1930), Kline (1930), Poe (1931), Pawan (1931), Ruchhoft *et al.* (1931) and Burke-Gaffney (1932) were among those who soon confirmed and extended Koser's work.

Braak (1928) made the first study of glycerol metabolism of "Intermediate" coliform bacteria and reported that such cultures converted glycerol into trimethylene glycol whereas control strains of the genera *Escherichia* and *Aerobacter* did not form the glycol. Werkman and Gillen (1932) confirmed the work of Braak and created the genus *Citrobacter* to include such intermediate strains.

Differential tests, in order to be completely useful, should be rapid, simple and easily executed. In the absence of such a test to determine the production of trimethylene glycol from glycerol, workers continued to use the methyl-red test, the Voges-Proskauer reaction and utilization of citrate for differentiation of the "Intermediate" coliform bacteria.

Fundamental physiological differences in the metabolism of glucose by the coliform bacteria have been recognized since Harden and his associates (1901, 1905, 1905–1906, 1911, 1911–1912) first showed that strains of *Aerobacter* decomposed glucose with the production of acetylmethylcarbinol and 2–3, butylene glycol as well as two or more volumes of CO_2 to one of H_2 and considerably less acetic, lactic and succinic acid, than *Escherichia coli* which did not form appreciable quantities of acetylmethylcarbinol or 2–3, butylene glycol, produced CO_2 and H_2 in a ratio of 1:1 and formed significantly larger quantities of the acids. Furthermore, cultures of *Aerobacter* produced much more ethyl alcohol

than acetic acid from glucose, whereas cultures of *Escherichia* formed these compounds in approximately equimolar quantities. Scheffer (1928) studied the glucose metabolism of the coliform "Intermediate" group and found their metabolic end-products similar to those of *Escherichia* as is shown in table 1.

The Voges-Proskauer reaction, a qualitative test for acetylmethylcarbinol and the methyl red test, a qualitative measure of the intensity and fate of acids produced, are therefore of most value for rapid primary differentiation of the coliform bacteria. The value of these tests, first shown by Levine (1916) to

TABLE 1

End-products of glucose metabolism by coliform bacteria (data from Scheffer, 1928)

PRODUCTS	ESCHERICHIA COLI		"INTERMEDIATE"		AEROBACTER AEROGENES	
	mM.	Per cent of sugar fermented	mM.	Per cent of sugar fermented	mM.	Per cent of sugar fermented
Carbon dioxide	42.3	11.1	31.8	10.9	338.0	39.3
Hydrogen	41.0	0.5	32.5	0 5	100.0	0.5
Lactic acid	78.1	41.9	59.8	41.8	23.6	5.6
Succinic acid	25.4	17 3	21.9	20 1	6.8	2.1
Formic acid	1.96	0.5		0	15 7	1 9
Acetic acid	41.8	14.9	25 8	12.1	3.5	0.5
Ethanol	41 5	11.4	26 1	9 3	139.5	17.0
Acetylmethyl carbinol		0		0	trace	0
2-3 butylene glycol		0		0	110.8	26.4
Total		97.6		94 7		93.3
Ratio CO_2/H_2	1 03:1.00		0.98:1.00		3.38:1 00	

correlate with differences in glucose metabolism, has been firmly established. Koser's citrate test is then used for further separation of the coliform bacteria into three groups:

TEST	V.-P.	M.R.	CITRATE
Escherichia	−	+	−
"Intermediate"	−	+	+
Aerobacter	+	−	+

Unfortunately, earlier investigation of the coliform bacteria was materially hindered by lack of a sensitive qualitative method for determination of the Voges-Proskauer reaction. Thus, some of the cultures diagnosed as representatives of the "Intermediate" section, in reality, belonged to the genus *Aerobacter*. That such was the case was clearly shown by Vaughn, Mitchell and Levine (1939). After finding that some cultures classified as belonging to the "Intermediate" section were really *Aerobacter* strains, it was anticipated that further investigation would show that *Aerobacter* cultures could form trimethylene glycol from glycerol. This was proven by Mickelson and Werkman (1940).

Most confusion in classifying the "Intermediate" group has resulted from a tendency to allocate *Aerobacter* cultures having very weak or negative Voges-Proskauer reactions to the "Intermediate" section without realizing that, of the two compounds, acetylmethylcarbinol and 2–3, butylene glycol, the latter predominates and the carbinol is sometimes found only in traces, even when estimated quantitatively. Also, acetylmethylcarbinol is decomposed by the coliform bacteria according to Linton, (1925); Paine, (1927); Williams and Morrow, (1928); and Tittsler, (1938). Furthermore, routine use of an incubation temperature of 37°C. for the coliform bacteria mitigates against detection of *Aerobacter* when using the Voges-Proskauer reaction as a criterion, as does too short or too long incubation. Further confusion has resulted from failure to recognize that the citrate test is a measure of the ability of the coliform bacteria to readily utilize the citrate radical as *a sole source of carbon in an otherwise inorganic medium.*

In a search for other characteristics which would be more satisfactory for separating the "Intermediate" group from true *Escherichia* and *Aerobacter*, several differential tests have been suggested including the production of hydrogen sulfide, utilization of cellobiose and indole formation in tryptophane broth.

Levine and his students, (1932) found that the "Intermediate" cultures investigated by them formed hydrogen sulfide in proteose peptone, ferric citrate agar and later (1934) reported that not all "Intermediate" cultures produced hydrogen sulfide in their medium. Finally, Vaughn and Levine, (1936) showed that the diagnostic value of the proteose peptone, ferric citrate agar medium for hydrogen sulfide production was dependent upon the concentration of agar contained in the medium. A concentration of 1.5 per cent agar was optimum for differential purposes. With higher concentrations of agàr, the number of positive cultures was materially reduced whereas, in a medium containing appreciably smaller amounts of agar the differential value was completely lost.

It is to be emphasized that hydrogen sulfide production is constant only for a constant set of conditions. Experience has shown that proteose peptone ferric-citrate medium with 1.5 per cent agar is the most satisfactory medium for use as a differential test for the "Intermediate" coli-like bacteria. Media with added sulphur-containing compounds or employing more sensitive indicators are as inadequate for good differentiation as liquid proteose peptone ferric-citrate medium in which it has been shown that the majority of strains of *Escherichia* and *Aerobacter* as well as the "Intermediate" coli-like cultures are capable of producing H_2S from the sulphur-containing constituents of the peptone.

The fermentation of cellobiose was first used by Jones and Wise (1926) and Koser (1926) with later confirmation by Skinner and Brudnoy (1932) and more recently by Tittsler and Sandholzer (1935), Stuart *et al.* (1938) and Mitchell and Levine (1938). Cellobiose fermentation will distinguish between true *Escherichia* and "Intermediate" cultures but it must be remembered that *Aerobacter* cultures attack cellobiose.

Other tests, particularly indole formation from tryptophane broth, have been used in an attempt to separate the coliform bacteria. Here, too, success has

been limited because of the high percentage of *Aerobacter* cultures which produce indole. Indole is a character better suited to specific than to generic differentiation.

As a result of the confusing and ambiguous results obtained with the differential tests cited above, some workers believed that part of the "Intermediate" organisms should be allocated to *Escherichia* and the rest to *Aerobacter* (Carpenter and Fulton, 1937). Others considered them all as *Escherichia* (Tittsler and Sandholzer, 1935; Yale, 1939); still others refused to allocate them but retained the "Intermediate" designation (Koser, 1924; Ruchhoft *et al.*, 1931; Bardsley, 1934; and many others); some gave them separate generic rank as *Citrobacter* (Werkman and Gillen, 1932; Bartram and Black, 1937) and some placed all of the coliform bacteria in one genus, *Bacterium* (Minkewitsch, 1930; Skinner and Brudnoy, 1932; Parr, 1938; and Malcolm, 1938).

Regardless of the manner in which the taxonomy of the group is finally treated, the "Intermediate" coliform bacteria are widely recognized and with more experience and more refined diagnostic methods, two alternatives are apparent: the "Intermediate" coliform bacteria must either be recognized as a separate genus or be given specific allocation in some existing genus. In either case, more attention must be given to specific and varietal differences.

The following information is presented with respect to the taxonomic status of the "Intermediate" coli-like bacteria; to differentiate them from *Aerobacter* and the characteristic strains of true *Escherichia* and to offer satisfactory species and varietal descriptions.

II. CHARACTERISTICS OF THE "INTERMEDIATE" COLI-LIKE BACTERIA

A. Source of cultures

The cultures to be considered are true "Intermediate" coli-like bacteria: gram-negative, short rods; do not produce spores; are generally motile with peritrichous flagellation; ferment lactose with acid and gas; do not produce acetylmethylcarbinol (V.-P. negative); and utilize citrates as a sole source of carbon (citrate positive).

The 223 cultures included in this investigation were obtained from a wide variety of sources including the feces of humans and fowls, various types of waters and soils, chicken eggs, milk, oysters and olives. The collection included cultures from laboratories in various parts of the world as well as strains isolated by the authors.

B. Methods

The cultures were all subjected to serial replating on Levine's eosine-methylene-blue agar for purification. Well isolated colonies were picked and purified by repeated plating from lactose broth (Standard Methods, 1936).

Primary differentiation from true *Escherichia* and *Aerobacter* was made on the basis of the Voges-Proskauer and Koser's citrate tests. The V.-P. reaction was determined by the use of Barritt's (1936) alpha-naphthol reagents on cultures grown in Difco M.R.-V.P. medium at 30°C. for periods of from one to

five days, (Vaughn, Mitchell and Levine, 1939). One ml. of culture was used to which was added 0.6 ml. of 5 per cent α-naphthol in *absolute ethyl alcohol* and 0.2 ml. of 40 per cent KOH. The V.-P. reactions, using Barritt's reagents, are best observed 30 minutes to 6 hours after addition of the reagents. On long standing, the crimson to ruby color denoting a positive reaction becomes darkened to brownish color which may turn very dark brown and form a heavy precipitate or return to a coppery color characteristic of a negative reaction.

The ability of the cultures to utilize the citrate radical as the sole source of carbon was determined by use of *Difco* Koser's citrate medium. Care was taken to exclude extraneous carbonaceous and nitrogenous contamination by acid washing and carefully rinsing all test tubes with distilled water. The medium was prepared with distilled water. Inoculations were made from 18- to 24-hour nutrient broth cultures using a straight needle.

Secondary or specific allocation, using the production of hydrogen sulfide from proteose peptone ferric citrate agar, the formation of indole in tryptophane broth, using Kovac's reagent (1928), the fermentation of starch, aesculin, salicin and glycerol followed the suggestion of Levine *et al.* (1934). These and other carbon compounds were added in concentrations of 0.3 to 0.5 per cent to a basal medium containing 5 grams of Bacto-peptone, 1 gram of K_2HPO_4 and 10 ml. of Andrade indicator per liter of distilled water.

The starch and aesculin media were prepared differently. Since it is important that the starch used be free from reducing substances, *Kingsford* and *Argo* corn starches known not to reduce Fehling's solution were used. A concentration of 1 per cent starch was prepared by making a thin paste in a small portion of cold basal medium and adding this paste to the desired quantity of boiling basal medium. With care a satisfactory suspension of the starch is obtained. *Soluble starches are unsatisfactory for differential purposes as most coliform bacteria readily ferment them,* since they contain products of starch hydrolysis.

The aesculin medium was prepared by adding 0.3 per cent aesculin and 0.05 per cent ferric citrate to the basal medium. Decomposition of the aesculin is denoted by blackening of the medium and accumulation of gas in the Durham tubes.

Motility was determined by hanging drop preparations of young cultures grown in nutrient broth and by the use of semi-solid agar (nutrient broth plus 0.5 per cent agar). Flagella stains, where made, were prepared according to the method of Plimmer and Paine (1921).

C. Differentiation of the "Intermediate" section

When it was recognized that an "Intermediate" group of coli-like organisms existed, primary differentiation from true *Escherichia* and *Aerobacter* cultures was made by the use of the Voges-Proskauer, methyl-red and Koser's citrate tests. Soon, however, *Aerobacter*-like cultures were isolated and classified as "Intermediate" coli-like bacteria and confusion reached such a state that some workers felt it would be desirable to allocate all "Intermediate" cultures to the two existing genera, depending upon which genus was more closely simulated.

As pointed out previously, part of the confusion arose through inadequate control of differential criteria. The fundamental differences in glucose metabolism were not widely recognized. Furthermore, a tendency to attempt to use

TABLE 2

Differentiation of sections of the coliform bacteria

CHARACTER	ESCHERICHIA	"INTERMEDIATE"	AEROBACTER
V.-P.	−	−	+
Citrate	−	+	+
M. R.	+	+	−
H_2S	−	+	−
Cellobiose	−	+	+
Uric acid (1)	−	−	+
Urea (1)	−	+	+
Yeast Nucleic acid (1)	−	−	+
Allantoin (1)	−	−	+
Hydantoin (1)	−	−	+
Uracil (1)	+	−	+
Growth at 45°–46°C. (2)	+	−	−
Resistance to H_3BO_3 (3)	+	−	−
Decomposition of Na malonate (4)	−	−	+
Metabolite specificity (5)	Specific for *Escherichia*	Specific for "Intermediate"	Specific for *Aerobacter*
Bacteriophage specificity (6)	Specific for *Escherichia*	Specific for "Intermediate"	Specific for *Aerobacter*
Origin	Predominant in vertebrate feces	Indefinite	Predominant in non-fecal materials

(1) Mitchell and Levine, (1938); West, Gililland and Vaughn (1941)
(2) Levine, Epstein and Vaughn (1934)
(3) Levine, (1921); Levine, Epstein and Vaughn, (1934); Vaughn and Levine, (1935); Vaughn, (1935)
(4) Leifson, (1933); West, Gililland and Vaughn (1941)
(5) Powers and Levine (1937)
(6) Powers, (1938); Powers, Levine and McCleskey (1938)

characters for generic diagnosis which are most useful for species or varietal differentiation still exists. What, then, are the limits of group differentiation?

The characters shown in table 2 appear particularly satisfactory for group differentiation.

On the basis of the differences shown in the table it is felt that there is sufficient evidence for regarding the true "Intermediate" coliform bacteria as a separate, distinct group. It is true that in some characteristics the "Intermediate" group appears more closely related to *Escherichia* as shown by the metabolic end-products of glucose fermentation as manifested by the negative Voges-Proskauer reaction and the positive methyl-red test for true *Escherichia.* On the other hand, characteristics suggestive of *Aerobacter* include the utilization of citrate as a sole source of carbon, failure to grow at 45–46°C., and low resistance to boric acid media.

The most outstanding characteristic possessed by the "Intermediate" section not common to *Escherichia* or *Aerobacter*, is the ability to form hydrogen sulfide from proteose peptone ferric-citrate agar.

Generic ranking of the "Intermediate" section has presented a confusing problem in the past. Purely on the basis of glucose metabolism the "Intermediate" bacteria are closely related to true *Escherichia* as has already been stressed. The genus *Citrobacter* as originally created for the "Intermediate" section was not satisfactory for it permitted the presence of *Aerobacter* types—*"acetoin not produced from glycerol and rarely from glucose and then only in traces."* Increase in our knowledge of the coliform bacteria and the advent of more refined techniques have, however, shown that the "Intermediate" group can be differentiated. Furthermore, the section has been widely recognized and specifically differentiated from *Escherichia* and *Aerobacter.* It would seem that steps should be taken to insure a legitimate taxonomic position for the "Intermediate" coliform group.

If the term *Citrobacter* is to be accorded generic rank, then the characterization by Mitchell and Levine (1938) should be given serious consideration. They point out as shown in table 2 that the "Intermediate" coli-like bacteria (*Citrobacter*) differ from true *Escherichia* cultures in that the former (1) utilize urea but not uracil as a sole source of nitrogen, (2) utilize citric acid and cellobiose as sole carbon sources, and (3) generally produce H_2S but not indole. Further evidence to strengthen the position of the "Intermediate" coli-like bacteria is also to be found in table 2 where it has been shown that the "Intermediate" coli-like bacteria differ from true *Escherichia coli* in that they (1) do not grow well at 45°–46°C. (Eijkman test), (2) are not resistant to 0.325 per cent boric acid in buffered lactose broth, (3) produce "metabolites" which inhibit a large proportion of intermediate strains but do not appreciably influence the growth of *Escherichia* or *Aerobacter* cultures, (4) exhibit marked group specificity for bacteriophages ("Intermediate" phages attack only "Intermediate" cultures), and (5) do not have a definite origin but are apparently quite evenly distributed in a wide variety of habitats (see table 6).

The genus *Citrobacter* (Werkman and Gillen) has not been satisfactory for allocation of the "Intermediate" coli-like bacteria. The physiological similarity between "Intermediate" coli-like cultures and true *Escherichia coli* cultures as indicated by the metabolism of glucose might be considered of sufficient magnitude to make the allocation of the "Intermediate" section to the genus *Es-*

cherichia mandatory. Such allocation, however, should be made with the understanding that the presence of "Intermediate" coli-like cultures in a food (as for example oysters or olives) is not to be regarded as *prima facie* evidence of fecal pollution. From the standpoint of sanitary significance, allocation of the "Intermediate" section to the genus *Escherichia* can not be completely satisfactory until the origin of the "Intermediate" coli-like organisms is conclusively proved. Evidence available at present does not indicate a clear cut solution of the question of the sanitary significance of "Intermediate" coli-like bacteria found in foods or in water.

The "Intermediate" coli-like bacteria possess the following general characteristics:

Gram-negative short rods; do not form spores; generally motile—if motile, with peritrichous flagellation; ferment lactose with production of acid and gas; ferment glucose with formation of equal volumes of CO_2 *and* H_2 *but no acetylmethylcarbinol (V.-P. negative) and form significant quantities of lactic, acetic and succinic acids (M.R.+); utilize citric acid as a sole source of carbon; generally produce* H_2S *in proteose peptone ferric-citrate agar; decompose cellobiose with the production of acid or acid and gas; utilize urea as a sole source of nitrogen but not yeast nucleic acid, uracil, uric acid, allantoin or hydantoin; do not grow well at 45°–46°C. (Eijkman test negative); fail to grow in boric-acid lactose broth at 42°–43°C.; have an optimum temperature range of 30° to 37°C.*

It is to be emphasized that with most of these characters, at least a few cultures in any of the three sections will be found to be in disagreement with the majority. Other characters, such as indole production, are not considered of significance for generic differentiation although very useful for species identification. (A large number of *Aerobacter* cultures produce indole.) All are conversant with the vagaries of the methyl-red reaction, and since when properly ascertained it correlates so well with the V.-P. reaction its use for differentiation is not pertinent.

D. Specific differentiation

Results of investigation on the carbon compounds decomposed by the "Intermediate" cultures is summarized in table 3.

Of the 27 compounds tested, 14 were attacked by all, or nearly all cultures, only 2 were not decomposed and 12 were considered of possible value for species differentiation.

It has already been shown that hydrogen sulfide production is a characteristic common to most of the "Intermediate" section. Experience has shown that this character correlates well with other reactions for the purposes of differentiating specific types of "Intermediate" bacteria.

Table 3 shows the specific differences between the H_2S positive and negative cultures. One hundred ninety-six cultures produced H_2S and only 27 cultures were negative. Of the 12 characteristics used in segregation of these two groups only a few were of significant differential value. The other carbon compounds decomposed were attacked at random by both groups and an attempt to em-

ploy all such characters would tend to increase the number of groups geometrically by the expression 2n where "n" is the number of characters studied. Obviously, such a practice would greatly confuse satisfactory grouping of the cultures.

Those characters thought to be of value for differentiation of the H_2S positive and negative types included indole production, motility, and fermentation

TABLE 3

Group differentiation of the 223 "Intermediate" Coli-like cultures

GROUP	ALL STRAINS (223 CULTURES)		H_2S POSITIVE (196 CULTURES)		H_2S NEGATIVE (27 CULTURES)	
	Positive reactions					
Character*	Number	Per cent	Number	Per cent	Number	Per cent
Indole	17	7.6	3	1.5	14	59.9
Motility	217	97.3	196	100.0	21	77.8
Starch	6	2.7	0	0.0	6	22.2
Aesculin	17	7 6	1	0.5	16	59.2
Salicin	43	19 3	24	12.2	19	70.4
Inositol	5	2.2	0	0.0	6	23.1
Glycerol	216	96 9	195	99.5	21	77.8
Sucrose	127	57.0	112	57.2	14	51.9
Raffinose	125	56.1	112	57.2	13	48.2
Melezitose	21	9 4	21	10.7	0	0.0
Dulcitol	94	42 2	88	44.9	6	22.2
Adonitol	4	17.9	0	0.0	4	14.8
α-methyl glucoside	86	38.6	75	38.3	11	40.8
Na-malonate	20	8.8	11	5.6	9	33.3
Trehalose	222	99.6	195	99.5	27	100.0
Sorbitol	220	98.7	195	99.5	25	92.6
Cellobiose	215	96.4	191	97.5	24	88.9

* Xylose, arabinose, rhamnose, glucose, galactose, fructose, mannose, lactose, maltose and mannitol attacked with acid or acid and gas production by all cultures. Amygdalin and erythritol not decomposed.

of starch, aesculin, salicin, inositol and glycerol. The coefficients of correlation[1] of these characters are shown in table 4.

The coefficients of correlation were calculated by formula I.

$$\frac{a(a+b+c+d)-(a+c)(a+b)}{\sqrt{(a+c)(b+d)(a+b)(c+d)}} \quad \text{(I)}$$

as suggested by Yule for situations where perfect correlations of 1 or −1 would result if any one group, a, b, c or d was zero. Otherwise, coefficients of corre-

[1] For a detailed discussion of this method consult Levine (1918).

lation for these characters may be erroneous in that perfect correlations of 1 or -1 are obtained for a large number of characters of little or no significance if the formula (II) $\frac{ad - bc}{ad + bc}$ is used.

For this study, using formula I, it has been considered that if the coefficient of correlation between two characters is greater than 0.5 they may be regarded as correlated, but if less than 0.3 there is probably no correlation.

Inspection of table 4 shows that H_2S production is significantly correlated with indole production and the decomposition of aesculin and to a lesser extent with motility and decomposition of inositol and salicin; the association being negative with the exception of motility.

All of the characters do not correlate with each other. Starch, which does not correlate well with H_2S has a high degree of association with motility and inositol but less significantly with aesculin. Indole production correlates well only with H_2S and aesculin. Glycerol is not correlated with any of the other characters. Aesculin shows a significant degree of association with all characters except glycerol but the number of positive reactions is very small. Motility correlates with starch, inositol, aesculin and H_2S. Inositol correlates particularly well with starch and motility and to a lesser degree with aesculin and H_2S. Salicin correlates only with aesculin and H_2S although the degree of association is not good.

TABLE 4

*Coefficients of correlation for each pair of significant characters using formula I**

	H_2S	INDOLE	STARCH	AESCULIN	SALICIN	INOSITOL	GLYCEROL	MOTILITY
H_2S		−0.62	−0.14	−0.83	−0.47	−0.45	−0.40	+0.50
Indole	−0.62		+0.16	+0.62	+0.42	+0.06	−0.09	−0.16
Starch	−0.14	+0.16		+0.58	+0.34	+0.83	+0.01	−0 83
Aesculin	−0.83	+0.62	+0 58		+0.60	+0.48	−0.14	+0.58
Salicin	−0.47	+0.42	+0.34	+0.60		+0.33	−0.04	−0.34
Inositol	−0.45	+0.06	+0.83	+0.48	+0.34		+0.03	−0.65
Glycerol	−0.40	−0.09	+0.01	−0.14	−0.04	+0.03		−0.03
Motility	+0.50	−0.16	−0.83	+0.58	−0.34	−0.65	−0.03	

* No significant correlation was found for the other characters.

Two specific groups are evidently represented by the 223 "Intermediate" cultures; a large H_2S positive group represented by 196 cultures and a small H_2S negative group of 27 cultures. The H_2S positive group is well defined. It rarely produces indole; does not ferment starch, aesculin or inositol; is motile and ferments glycerol.

The H_2S negative group is quite heterogeneous. Inspection of the results shown in table 3 indicated that two types or varieties might be present among the H_2S negative cultures. This supposition was tested by determining the coefficients of correlation among the same characters as used for group differentiation. The coefficients of correlation are shown in table 5. There is a high

degree of association between starch, inositol and motility. The other characters do not correlate well with the exception of aesculin with salicin and to a lesser extent indole with salicin. Two types are present; one, represented by 6 cultures, ferments starch and inositol but is non-motile; the other, comprising 21 cultures, does not ferment starch or inositol but is motile. (Malcolm, 1938, has also found inositol an aid for specific differentiation of the "Intermediate" coli-like bacteria as indicated by his correlation studies.)

TABLE 5

Coefficient of correlation for each pair of significant characters of the H_2S negative cultures, using formula I

	INDOLE	STARCH	AESCULIN	SALICIN	INOSITOL	GLYCEROL	MOTILITY
Indole		−0.20	+0.40	+0.51	−0.38	+0.38	+0.20
Starch	−0.20		+0.44	+0.35	+0.78	+0.29	−0.79
Aesculin	+0.40	+0.44		+0.78	+0.26	+0.21	−0.44
Salicin	+0.51	+0.35	+0.78		+0.58	+0.47	−0.35
Inositol	−0.38	+0 78	+0 26	+0.58		+0.28	−0.57
Glycerol	+0.38	+0.29	+0.21	+0 47	+0.28		−0.29
Motility	+0.20	−0 79	−0.44	−0.35	−0.57	−0.29	

The "Intermediate" section may be divided into three well defined groups as follows:

V.-P. −
M. R. +
Citrate +
Cellobiose +

H_2S +
Indole −
Starch −
Aesculin −
Inositol −
Glycerol +
Motility +
(196 cultures)

H_2S −

Starch +
Inositol +
Motility −
(6 cultures)

Starch −
Inositol −
Motility +
(21 cultures)

Two sections should be recognized among the "Intermediate" coli-like bacteria, an H_2S positive group which is well defined and an H_2S negative group which may be further satisfactorily differentiated. In some other collection of cultures the H_2S negative group might be the larger. Representatives of both groups have been described by many investigators. The two groups should be allocated to the genus *Escherichia*.

As already pointed out, the ability of the "Intermediate" coli-like bacteria to produce hydrogen sulfide in proteose peptone ferric-citrate agar is a characteristic distinctive for most of the cultures of this section of coliform bacteria. For adequate differentiation it is desirable to recognize at least two definite

groups because of the distinct differences between the hydrogen sulfide positive and negative cultures. The large H_2S+group of "Intermediate" coli-like bacteria might be recognized as *Escherichia freundii* (Braak) Yale. The small H_2S-group might be recognized as *Escherichia intermedium* (Werkman and Gillen) *nov. comb.*

Description of Escherichia intermedium. (Werkman and Gillen) nov. comb.

Morphology (at 30°).

Form and arrangement:—Short rods with rounded ends which occur singly, in pairs and short chains when young nutrient agar or broth cultures are examined.

Spore formation:—No spores have been observed.

Motility:—Actively motile with peritrichous flagella or non-motile.

Staining reactions:—Gram-negative.

Cultural characteristics (at 30°C)

Nutrient agar:—Growth is abundant, raised, greyish white with smooth to wrinkled surface and butyrous consistency.

Nutrient broth:—An abundant turbidity is formed with surface growth confined to slight ring formation.

Gelatin stab (at 20°C):—Growth along the line of inoculation but no liquefaction after 60 days.

Potato:—Growth on potato wedges is abundant with a white to ivory color.

Litmus milk:—The milk is acidified; sometimes followed by coagulation and reduction. Gas is formed. Proteolysis has not been observed.

Levine's eosine-methylene-blue agar:—Well isolated colonies vary from 1 to 4 mm in size. There is no confluence of neighboring colonies so characteristic for cultures of *Aerobacter*. Colonies are slightly to moderately raised with surfaces varying from flat to convex and usually smooth and glistening but sometimes dull, rough and granular.

By transmitted light two types of colonies have been observed: 1) Colonies having almost the same appearance throughout but with a distinctly lighter center, the color being similar to the medium. 2) Colonies having a dark brownish central area which diffuses out to a lighter margin.

By reflected light three types of colonies have been observed: 1) Dark, button-like, concentrically ringed colonies possessing a strong greenish metallic sheen so characteristic for *Escherichia coli*. 2) Colonies with dark, purplish, wine-colored centers surrounded by a light pink zone. Some colonies are concentrically ringed. 3) Pink colored colonies with no suggestion of sheen but sometimes concentrically ringed. The two latter types predominated among the cultures studied.

Biochemical characteristics (at 30°C)

Catalase:—This enzyme is produced by all cultures.

Fermentation of glucose:—The end-products characteristic for the genus

Escherichia are formed. Carbon dioxide and hydrogen gases are formed in approximately equimolar proportions (*gas ratio 1:1*) besides significant quantities of ethanol and acetic, lactic and succinic acids (*methyl-red test positive*) with only traces of formic acid. Acetylmethylcarbinol and 2–3 butylene glycol have not been found (*Voges-Proskauer test negative*).

Citrate utilization:—Salts of citric acid are utilized as a sole source of carbon in an otherwise inorganic medium.

Hydrogen sulfide:—Not detected in proteose peptone ferric-citrate agar.

Indole:—May or may not be formed in tryptophane broth cultures.

Nitrates:—Reduced to nitrites.

Xylose, arabinose, rhamnose, glucose, fructose, mannose, galactose, lactose, maltose, trehalose and mannitol decomposed with acid or acid and gas production by all cultures. Melezitose, amygdalin and erythritol not attacked.

Sucrose, raffinose, cellobiose, α-methyl glucoside, adonitol, dulcitol, glycerol, inositol, sorbitol, starch, aesculin, salicin and sodium malonate attacked by some cultures and not by others.

Requirements for growth

Oxygen relationships:—Facultative.

Temperature requirements:—Growth occurs at 10°C and at 45° to 46°C. The optimum temperature for growth in the media used for study is between 30° and 37°C. Although some cultures show growth at 45° to 46°C gas is not produced in Eijkman test media when incubated at 43° to 46°C (temperature of the medium).

Salt tolerance:—Most cultures ferment glucose in the presence of sodium chloride in a concentration of 6.0 to 7.0. A few cultures tolerate 8.0 per cent sodium chloride.

pH range:—Cultures grow best in nearly neutral media although growth has been observed at pH 5.0 and pH 8.0.

Distinguishing characteristics

Cultures of *Escherichia intermedium* are differentiated from *Escherichia freundii* by the lack of ability of the former to produce hydrogen sulfide in proteose peptone ferric-citrate agar.

Two varieties of *Escherichia intermedium* have been observed. One was composed of 21 cultures which were unable to decompose starch or inositol but were motile. The other consisted of 6 cultures which decomposed starch and inositol with formation of acid and gas but were non-motile.

As already stressed it is not desirable to separate the H_2S negative *Escherichia intermedium* group into two varieties until a much larger number of such isolates have been available for study.

Sources of isolates

Cultures of *Escherichia intermedium* have been isolated from water, feces, soil, milk and olives.

The characteristics which separate *Escherichia intermedium* and *Escherichia freundii* from true *Escherichia* (citrate negative) and *Aerobacter* have already been given in table 2. Cultures of *Bacterium freundii* (Braak) received from Professor Kluyver include both sulfide-producing and non-sulfide producing types. The marked differences between the original cultures make it advisable to give specific names to the two types, so that the differences may be more easily noted.

The question of proper synonymy is difficult. Certainly "Intermediate" coli-like bacteria had been described under a variety of species of *Escherichia*, *Bacterium* or *Bacillus* for some time prior to the work of Koser (1923), Braak (1928), and Werkman and Gillen (1932).

Until descriptions included utilization of citrate as a sole source of carbon, the production of hydrogen sulfide in peptone, lead acetate agar offered the best clue as to whether the organisms might belong to the "Intermediate" section provided that other reactions were characteristic for coli-like bacteria. A study of the literature shows that it would be very difficult, if not impossible, to establish synonymy in this manner.

The most probable synonyms are those species of *Citrobacter* named by Werkman and Gillen (1932) including *Citrobacter album*, *Citrobacter anindolicum*, *Citrobacter decolorans*, *Citrobacter diversum*, *Citrobacter glycologenes* and *Citrobacter intermedium*. Had the trinomial *Bacterium coli citrovorum* (Minkewitsch, 1930) been used as a binomial as *Escherichia citrovorum* the "Intermediate" coli-like bacteria would have been given a very descriptive name.

It is not desirable at this time to separate the H_2S negative group into two varieties. A much larger number of strains should be available for study before stressing subdivision on statistical correlation.

E. Some ecological implications

Although it has been claimed that the "Intermediate" coliform bacteria (genus *Citrobacter*) are probably not normal inhabitants of the vertebrate intestine, it should be pointed out that such a conclusion is not warranted.

The data summarized in table 6 have been compiled from the literature for the purpose of ascertaining in part the relative incidence of the "Intermediate" section among the coliform bacteria isolated from different sources and also for pointing out the confusion which accompanies the presence of this group when isolated in large numbers from various sources. The results are based upon differentiation through the use of the methyl-red, Voges-Proskauer and Koser's citrate tests.

The data in table 6 indicate that the "Intermediate" section is widely distributed in nature; that it is encountered only in small numbers in the feces of man and animals; that the question of sanitary significance of this group is difficult to ascertain; that on the basis of the available data there is a somewhat higher incidence of this type in soils considered to be remotely polluted but the percentage incidence is low in most cases, showing little correlation with source and with relatively small percentage differences in incidence from various sources,

the conclusion that the "Intermediate" group is necessarily non-fecal in origin is unjustifiable.

Part of the somewhat higher percentage incidence among the non-fecal sources may be accounted for by the fact that with a predominance of *Aerobacter* forms there would be a larger number of such cultures incorrectly diagnosed as "Intermediate" because of the use of unreliable techniques for determining the V.-P. and particularly the methyl-red reactions, as for example the tendency to incubate at 37°C.

TABLE 6

Summary of incidence of "Intermediate" Coliform bacteria

SOURCE	NUMBER OF CULTURES	SECTIONS OF COLIFORM BACTERIA							
		Escherichia		Aerobacter		"Intermediate"		Unclassified	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Feces	5010	4308	86.0	273	5.4	354	7.1	75	1.5
Urine	453	235	71.7	172	38.0	46	10.3	0	0
Unpolluted soil	527	54	10.3	400	75.9	63	11.9	10	1.9
Remotely polluted soil	340	89	26.2	173	50.9	72	21.2	6	1 7
Recently polluted soil	1698	430	25 3	1078	63.5	146	8.6	44	2.6
Miscellaneous soils*	799	164	20.5	468	58.6	142	17.8	25	3 1
All soil isolations	3364	737	21 9	2119	63 0	423	12.6	85	2.5
Unpolluted water	4209	1441	34 2	1897	45 0	616	14.7	255	6.1
Polluted water	2452	1441	58 7	707	28.8	190	7.8	114	4 7
Miscellaneous waters*	10482	5880	56.1	2283	21.8	1694	16.2	625	5.9
All water insolations	17143	8762	51.1	4887	28.5	2500	14.6	994	5 8
Milk	2316	661	28 5	1147	49.5	470	20.3	38	1.7
Dairy products	195	88	45.1	81	41.5	26	13.3	0	0
Eggs	401	155	38.7	203	50.6	43	10.7	0	0
Hay and grain	723	114	15.8	532	73.6	64	8.8	13	1.8
Olives	156	0	0	110	70.5	46	29.5	0	0

* Not possible to differentiate type of water or soil from which cultures were isolated.

III. DISCUSSION

The "Intermediate" cultures herein reported were collected and studied over a ten-year period. During this time, no unusual characteristic irregularities or "variants" have been noted except those which were discounted as due to previous incorrect allocation. As already emphasized, use of inadequate methods of determining the V.-P. reaction and the use of 37°C rather than 30°C for incubation accounted for most of the "variants."

It is important to note, however, that in one instance true *Escherichia coli* cultures had been previously diagnosed as "Intermediate" because Simmon's citrate agar made with tap water had been used for differential purposes. It is known that agar contains growth-promoting factors. Koser's citrate medium carefully prepared to avoid chemical contamination should be used for determining whether coliform cultures can use the citrate radical as a *sole source* of carbon.

IV. SUMMARY AND CONCLUSIONS

The "Intermediate" group consistutes a distinct section of the coliform bacteria and may be differentiated from both *Escherichia* and *Aerobacter*.

The "Intermediate" section differs from true *Escherichia* in that the former (1) utilizes the citrate radical as a sole source of carbon in an otherwise inorganic medium, (2) produces H_2S in proteose peptone, ferric citrate agar, (3) decomposes cellobiose, (4) uses urea as a sole source of nitrogen but does not use uracil, (5) does not grow well at 45°–46°C (Eijkman test) and (6) is not resistant to boric acid.

The "Intermediate" section may be differentiated from true *Aerobacter* in that the former (1) does not produce acetylmethylcarbinol from glucose (V.-P.), (2) is methyl-red positive, (3) generally produces H_2S in proteose peptone, ferric citrate agar, (4) does not utilize uric acid, uracil, yeast nucleic acid, allantoin or hydantoin as sole sources of nitrogen, but does use urea, and (5) does not decompose sodium malonate.

The "Intermediate" coli-like bacteria are markedly group specific in their reaction to bacteriophages and to metabolites from staled agar, as shown by the work of Powers and Levine.

The "Intermediate" bacteria are most closely related to the organisms of the genus *Escherichia* from the standpoint of the physiological similarity in the metabolism of glucose. For this reason the cultures studied were allocated to the genus *Escherichia*, stressing, however, that until the question of natural habitat of the "Intermediate" section has been more conclusively proved it is desirable to segregate such cultures from true *Escherichia coli* which is conceded to predominate in the feces of vertebrates.

On the basis of statistical correlation, two species of "Intermediate" coli-like bacteria were recognized. The predominant species, *Escherichia freundii*, was represented by 196 cultures. The remaining 27 cultures were described as *Escherichia intermedium nov. comb.* A new description of *Escherichia intermedium* is also given.

REFERENCES

American Public Health Association. 1936 Standard methods for the examination of water and sewage. 8th edition, American Public Health Association, New York.

Bardsley, Doris. 1926 *B. coli* as an index of faecal pollution of water supplies. J. Hyg., **25**, 11–25.

Bardsley, Doris A. 1934 The distribution and sanitary significance of *B. coli*, *B. lactis aerogenes* and intermediate types of coliform bacilli in water, soil faeces and ice-cream. J. Hyg., **34**, 38–68.

Barritt, M. W. 1936 The intensification of the Voges-Proskauer reaction by the addition of alpha-naphthol. J. Path. Bact., **42**, 441–454.

Bartram, M. T., and Black, L. A. 1937 Detection and significance of the coliform group in milk. II. Identification of species isolated. Food Research, **2**, 21–26.

Braak, H. R. 1928 Onderzoekingen over vergisting van glycerine. Thesis, Delft, Holland.

Brown, J. W., and Skinner, C. E. 1930. Is the Eijkman test an aid in the detection of of fecal pollution of water? J. Bact., **20**, 139–150.

Burke-Gaffney, H. J. O'D. 1932 The classification of the colon-aerogenes group of bacteria in the sanitary examination of water supplies in the tropics and in temperate climates. J. Hyg., **32**, 85–131.

Carpenter, P. L., and Fulton, MacD. 1937 Escherichia-Aerobacter intermediates from human feces. Am. J. Pub. Health, **27**, 822–827.

Griffin, A. M., and Stuart, C. A. 1940 An ecological study of the coliform bacteria J. Bact., **40**, 83–100.

Harden, A. 1901 The chemical action of *Bacillus coli communis* and similar organisms on carbohydrates and allied compounds. J. Chem. Soc., **79**, 610–628.

Harden, A. 1905 Action on glucose of lactose fermenting bacteria of feces. J. Hyg., **5**, 488–493.

Harden, A., and Walpole, G. S. 1905–1906 Chemical action of *B. lactis aerogenes* (Escherich) on glucose. Production of 2–3 butylene glycol and acetylmethyl carbinol. Proc. Roy. Soc. (London), B, **77**, 399–405.

Harden, A. 1905–1906 On the Voges-Proskauer reaction for certain bacteria. Proc. Roy. Soc. (London), B, **77**, 424–425.

Harden, A., and Norris, D. 1911 The diacetyl reaction for proteins. J. Physiol., **47**, 332–336.

Harden, A., and Norris, D. 1911–1912 Bacterial production of acetyl methyl carbinol and 2–3 butylene glycol from various substances. Proc. Roy. Soc. (London), B, **84**, 492–499; **85**, 73–78.

Holwerda, K. 1928 Some experiments on the changes in the bacterial flora of stored polluted surface water, especially with a view to Clemesha's method for ascertaining the recency of a pollution. Mededeel. Dienst Volksgezondheid Nederland.-Indië, **17**, 298–356 (Foreign Edition).

Holwerda, K. 1930 Research into the properties of the aerobe, the nonsporulating-lactose fermenting bacteria in feces and soil in the tropics and the importance thereof for the analysis of drinking water. Mededeel. Dienst Volksgezondheid Nederland.-Indië, **19**, 289–324.

Hill, J. H., Seidman, L. R., Stadnichenko, A. M. S., and Ellis, M. G. 1929 A study of 200 cultures of gram-negative bacilli isolated from cases of genito-urinary infection. J. Bact., **17**, 205–246.

Jones, H. N., and Wise, L. E. 1926 Cellobiose as an aid in the differentiation of members of the colon-aerogenes group of bacteria. J. Bact., **11**, 359–366.

Kline, E. K. 1930 The colon group of bacteria in milk. Ann. Rept. Intern. Assoc. Dairy and Milk Inspectors, **19**, 68–85.

Kline, E. K. 1935 Constancy of characters differentiating intermediates in the *Escherichia Aerobacter* group and their interpretation. Am. J. Pub. Health, **25**, 833–838.

Koser, S. A. 1923 Utilization of the salts of organic acids by the colon-aerogenes group. J. Bact., **8**, 493–520.

Koser, S. A. 1924 Correlation of citrate utilization by members of the colon-aerogenes group with other differential characteristics and with habitat. J. Bact., **9**, 59–77.

Koser, S. A. 1926 Cellobiose fermentation by the coli-aerogenes group. J. Infectious Diseases, **38**, 506–510.

Koser, S. A., and Saunders, F. 1932 The fermentation of alpha-methyl-d-glucoside by members of the coli-aerogenes group. J. Bact., **24**, 267–271.

KOVACS, N. 1928 Eine vereinfache Methode zum Nachweis der Indolbildung durch Bakterien. Z. Immunitäts., **55**, 311–315.

LEIFSON, EINAR. 1933 The fermentation of sodium malonate as a means of differentiating *Aerobacter* and *Escherichia*. J. Bact., **26**, 329–330.

LEVINE, MAX. 1916 The correlation of the Voges-Proskauer and methyl-red reactions in the colon-aerogenes group of bacteria. J. Infectious Diseases, **18**, 358–367.

LEVINE, MAX. 1918 A statistical classification of the colon-cloacae group. J. Bact., **3**, 253–276.

LEVINE, MAX. 1921 Notes on *Bact. coli* and *Bact. aerogenes*. Am. J. Pub. Health, **11**, 21–23.

LEVINE, MAX, VAUGHN, REESE, EPSTEIN, S. S., AND ANDERSON, D. 1932 Some differential reactions in the colon-aerogenes group of bacteria. Proc. Soc. Exptl. Biol. Med., **29**, 1022–1024.

LEVINE, MAX, EPSTEIN, S. S., AND VAUGHN, R. H. 1934 Differential reactions in the colon group of bacteria. Am. J. Pub. Health, **24**, 505–510.

LEWIS, I. M., AND PITTMAN, E. E. 1928 Correlation between differential tests for colon bacteria and sanitary quality of water. J. Am. Water Works Assoc., **19**, 78–91.

LINTON, C. S. 1925 A note on the Voges-Proskauer reaction. J. Am. Water Works Assoc., **13**, 547–549.

MALCOLM, JAMES F. 1938 The classification of coliform bacteria. J. Hyg., **38**, 395–423.

MICKELSON, M. N., AND WERKMAN, C. H. 1940 Formation of trimethylene glycol from glycerol by *Aerobacter*. Enzymologia, **8**, 252–256.

MINKEWITSCH, I. E., TROFIMUK, N. A., AND WEDENJAPIN, S. A. 1928 Über die Bedeutung der Zweifachen Herkunft der Bakteriengruppe Coli-aerogenes für die sanitäre Beurteilung des Trinkwassers. Z. Hyg. Infektionskrankh., **109**, 348–353.

MINKEWITSCH, I. E. 1930 Die Grundtypen der Bakteriengruppe Coli-aerogenes und ihre Herkunft. Z. Hyg. Infektionskrankh., **111**, 180–190.

MITCHELL, N. B., AND LEVINE, MAX. 1938 Nitrogen availability as an aid in the differentiation of bacteria in the coli-aerogens group. J. Bact., **36**, 587–598.

OESER, H. 1937 *Bacterium coli* in der Milch. Zentr. Bakt. Parasitenk. II., **96**, 287–329.

PAINE, F. S. 1927 The destruction of acetyl methyl carbinol by members of the colon-aerogenes group. J. Bact., **13**, 269–274.

PARR, LELAND W. 1938 Coliform intermediates in human feces. J. Bact., **36**, 1–15.

PAWAN, J. L. 1931 Some aspects of the colon group of organisms with special reference to the water supplies of Trinidad. J. Trop. Med. Hyg., **34**, 229–236; 267–274; 310–313; 317–327; 345–348; 360–363; 380–383; 391–395; 413–416.

PLIMMER, H. G., AND PAINE, S. G. 1921 A new method for the staining of bacterial flagella. J. Path. Bact., **24**, 286–288.

POE, CHARLES, F. 1931 Differential tests for the colon-aerogenes group. J. Am. Water Works Assoc., **23**, 1218–1226.

POWERS, M. J. 1938 Bacteriophage specificity for coliform and related bacteria. Iowa State College J. Sci., **13**, 88–90.

POWERS, M. J., AND LEVINE, MAX. 1937 Effect of metabolites on growth and differentiation in the colon-group. Proc. Soc. Exptl. Biol. Med., **36**, 274–276.

POWERS, M. J., LEVINE, MAX, AND MCCLESKEY, C. S. 1938 Specificity of bacteriophage for strains of the *Escherichia-Aerobacter* group and for certain other members of the *Bacteriaceae*. J. Bact., **36**, 295–296.

RAGHAVACHARI, T. N. S. 1926 The application of Koser's citrate utilization tests to sanitary water analysis in India. Indian J. Med. Research, **14**, 47–51.

RUCHHOFT, C. C., KALLAS, J. G., CHINN, BEN, AND COULTER, E. W. 1931 Coli-aerogenes differentiation in water analysis. J. Bact., **21**, 407–440.

RUCHHOFT, C. C., KALLAS, J. G., CHINN, BEN, AND COULTER, E. W. 1931 Coli-aerogenes differentiation in water analysis. II. The biochemical differential tests and their interpretation. J. Bact., **22**, 125–181.

Scheffer, M. A. 1928 De Suikervergisting door Bacteriën der Coli-Groep. Thesis, Delft.

Skinner, C. E., and Brudnoy, H. G. 1932 The utilization of citrates and the fermentation of cellobiose by strains of *Bacterium coli* isolated from human faeces. J. Hyg., **32**, 529–534.

Stuart, C. A., Griffin, A. M., and Baker, Muriel E. 1928 Relationships of coliform organisms. J. Bact., **36**, 391–410.

Taylor, J., Martin, C de C., Naidu, J. V. R., and Naidu, P. N. R. 1927 A comparison of the results of Clemesha's method and the test of citrate utilization as applied to water supplies in Burma. Indian J. Med. Research, **14**, 801–836.

Tittsler, R. P. 1938 The fermentation of acetyl methyl carbinol by the *Escherichia-Aerobacter* group and its significance in the Voges-Proskauer reaction. J. Bact., **35**, 157–162.

Tittsler, R. P., and Sandholzer, L. A. 1935 Studies on the *Escherichia-Aerobacter* intermediates. I. Cultural characteristics. J. Bact., **23**, 167–182.

Vaughn, Reese H. 1932 Organisms of the colon-aerogenes group utilizing citrate and producing hydrogen sulfide. Unpublished Thesis, Library, Iowa State College, Ames, Iowa.

Vaughn, Reese H. 1935 Selectivity of boric acid media in the colon group. Unpublished Thesis, Library, Iowa State College, Ames, Iowa.

Vaughn, Reese, and Levine, Max 1935 Effect of temperature and boric acid on gas production in the colon group. J. Bact., **29**, 24–25.

Vaughn, Reese, and Levine, Max 1936 Hydrogen sulfide production as a differential test in the colon group. J. Bact., **32**, 65–73.

Vaughn, Reese, Mitchell, N. B., and Levine, Max 1939 The Voges-Proskauer and methyl red reactions in the coli-aerogenes group. J. Am. Water Works Assoc., **31**, 993–1001.

Werkman, C. H., and Gillen, G. F. 1932 Bacteria producing trimethylene glycol. J. Bact., **23**, 167–182.

West, Nat. S., Gililland, J. Richard, and Vaughn, Reese H. 1941 Characteristics of coliform bacteria from olives. J. Bact., **41**, 341–352.

Williams, O. B., and Morrow, M. B. 1928 The bacterial destruction of acetyl methyl carbinol. J. Bact., **16**, 43–48.

Yale, M. W. 1933 The *Escherichia-Aerobacter* group of bacteria in dairy products. J. Dairy Sci., **16**, 481–494.

Yale, M. W. 1939 Bergey's Manual of Determinative Bacteriology, 5th ed., Williams & Wilkins Co., Baltimore, 394.

NOTES

RAPID DEMONSTRATION OF HEMOLYSIS DUE TO ANAEROBES

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In 1942 Brewer described a glass Petri dish cover and an agar which could be used without an anaerobic jar to obtain surface colonies of anaerobes. To investigate the usefulness of this procedure in the detection of hemolysis by anaerobic organisms, a series of experiments was performed with *Clostridium botulinum*, *C. histolyticum*, *C. novyi*, *C. perfringens*, *C. septicum*, *C. sporogenes*, *C. tetani* and two strains of streptococci.

"Anaerobic agar"[1] containing thioglycollate and sulfoxylate, with and without glucose, and with and without methylene blue, and ordinary infusion agar were used in Petri and Brewer plates. The media were cooled to 50°C. before the addition of blood; defibrinated rabbit and sheep blood and citrated human blood were employed with good results. The inoculum and 0.5 to 0.8 ml. of blood were mixed with 15 ml. of each medium and, when this had hardened in the bottom of a Petri dish, it was covered with 10 to 25 ml. of the same or different agar to make a seal with a Brewer lid. Brewer lids were substituted for the covers of half of the plates. In 10 ml. dishes with Brewer lids, the second layer was unnecessary when a little more blood agar was used. To obtain surface colonies the fluid blood-agar mixture was inoculated and poured over a layer of agar without blood, or the blood agar was allowed to harden and was then streaked. Cultures were incubated at 37.5°C.

The most satisfactory medium was a modified agar having the formula: polypeptone, 20 g.; sodium chloride, 5 g.; sodium thioglycollate, 2 g.; sodium formaldehyde sulfoxylate, 1 g.; agar, 20 g.; and water, 1000 ml. The medium induced rapid growth and hemolysis when used alone or as a second layer over an infusion blood-agar preparation. It was necessary to inoculate lightly so as not to obscure the pattern of hemolysis. Properly prepared plates had a light rose-red color instead of the orange-red of oxyhemoglobin and spectroscopic examination of the plates revealed the absorption spectrum of hemoglobin.

In the presence of reducing substances, visible growth and hemolysis appeared in 18 to 48 hours, usually within 24 hours. In preparations made entirely with infusion agar, only *C. sporogenes* and *C. histolyticum*, of the bacilli studied, grew as readily as on agar with special reducing agents. Agar with glucose was unsatisfactory for some species because it enhanced gas production.

In Brewer plates prepared with the modified agar, one or more characteristic zones of hemolysis formed around colonies. Poured plates gave the best results, and *C. novyi* and *C. perfringens* developed more elaborate patterns than

[1] Supplied by the Baltimore Biological Laboratory.

indicated by Reed and Orr (1941). On streaked plates surface colonies grew well and hemolysis occurred, but the zoning was less definite.

In Petri plates subsurface colonies showed true hemolysis and, usually later, green areas also developed. The color of the medium in regions unaffected by the colonies gradually changed from the "Burgundy red" to a more crimson shade. The nature of these changes is now being investigated.

REFERENCES

Brewer, J. H. 1942 A new Petri dish cover and technique for use in the cultivation of anaerobes and micro-aerophiles. Science, **95**, 587.

Reed, G. B., and Orr, J. H. 1941 Rapid identification of gas gangrene anaerobes. War Medicine, **1**, 493–510.

CHROMOBACTERIUM VIOLACEUM, VAR. MANILAE AS A PATHOGENIC MICROÖRGANISM[1]

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A definite and rapidly fatal infection of the human subject with *Chromobacterium violaceum, var. manilae* afforded the culture concerned in the present study. It is noteworthy that heretofore there have appeared in the literature only two reports concerning infection of the human subject and one of several carabao or water-buffalo with the microörganism usually termed *Bacillus violaceus*. It is of additional interest that all such infections in man or animal have sooner or later proved fatal to the invaded host.

It seems probable that *C. violaceum* has, in general, been erroneously considered as a distinctly non-pathogenic saprophyte because of lack of regard for group differentiation. Without proper recognition of variance for separate species as regards pathogenicity, it becomes difficult to comprehend that a supposedly innocuous chromobacterium should rarely become not only pathogenic but also possessive of death-producing virulence. Because of such facts, it appears in order to present briefly the salient features of the present day knowledge appertaining to *C. violaceum*.

THE MICROÖRGANISM

This organism, a gram-negative, motile bacillus, first described by Schroeter in 1872, represents the type species of the genus *Chromobacterium* of Bergonzini (1881). A complete description of this group of microorganisms is given by Ford (1927). In as much as those working in the field of pathogenic bacteria have little if any knowledge of the many allied saprophytic varieties of this group, a review of this subject as presented by Ford will be found of value.

The particular strain studied by us conforms, in general, to that described by Woolley (1905) in an indefensible trinomial form as "*B. violaceus Manilae.*" The morphological and biological characteristics of this bacillus will not be recounted in detail. Contrary to many members of this group, the optimum temperature of cultivation is 37°C. Upon potato media this organism presents a profuse rich violet growth, the purple pigment diffusing into the water of condensation. Its strong aerophilic proclivity is most striking as demonstrated by its surface flotation growth on tubed liquid media and heavy surface growth upon stab culture. Only scant, non-pigmented growth occurs along the stab beneath the surface. It liquefies gelatin to only a slight extent. Certain biochemical reactions upon various carbohydrate serum waters and pigment solubility in alcohol are quite consistent. As for other strains wherein the microör-

[1] Aided by a grant from the David Trautman Schwartz Research Fund.

ganism was of proven pathogenicity, no evidence of definite soluble or exotoxin production could be demonstrated.

The generally accepted opinion regarding the non-pathogenic and saprophytic nature of "*B. violaceus*" forms a distinct paradox to the high degree of pathogenicity for laboratory animals manifested by the cultures isolated from the few reported human cases.

DISEASE PRODUCTION

As stated, the instances on record wherein *C. violaceum* has demonstrated a definite pathogenic role are very limited. They consist of observations upon lower animal infection and in addition, two human invasions. These may be briefly summarized in chronological order:

In 1905 Woolley, described several fatal cases of infection in carabao or water-buffalo in Manila. These animals died suddenly with symptoms of acute hemorrhagic septicemia. Necropsy showed enlarged injected lymph glands and the lungs were studded with small firm gray nodules. *C. violaceum* was recovered from cultures of the lymph nodes and lungs. Since he could find no reference in the literature to the pathogenicity of *C. violaceum*, Woolley carried out rather extensive animal experimentation. He succeeded in reproducing the lesions of the carabao in various types of small animals by inoculation of the microörganism and was able to recover it in pure and abundant culture from the lesions and the heart's blood. He did not find evidence of any soluble toxin production for his strain. He termed this microörganism "*B. violaceus Manilae.*"

Black and Shahan (1937), in Florida, reported what was apparently the first instance of definite infection with *C. violaceum* in man. The case was that of a six-year-old boy in whom *C. violaceum* was recovered from persistent "anthrax-like lesions" of the trunk and upper extremities.

This patient apparently had been perfectly well until the onset of his infection. He had waded in a pool two or three days previously. His first complaint was pain in the inguinal region and he presented later an acute inguinal adenitis. There eventually occurred multiple "anthrax-like lesions" over the trunk and upper extremities which were surrounded by a pustular ring showing a distinct violet color. Smears from these lesions showed a slender gram-negative bacillus. Culture on agar produced deep violet colonies. Agglutination tests were positive with dilutions to 1–1280. One rabbit was injected subcutaneously with a bouillon culture and died 48 hours later. *C. violaceum* was recovered in pure culture from the liver, lungs, spleen, kidneys, heart's blood, and site of inoculation of the animal. Following a stormy course the patient's skin lesions finally healed and he apparently recovered. It is to be noted, however, in a subsequent report of Soule (1939) in referring to this child that he reappeared thirteen months later with severe cervical adenitis, high fever and prostration. The adenitis abated and the body became covered with lesions varying from minute vesicles to large gangrenous areas. The boy died fifteen months after the original onset of his illness or thirteen months after the apparent healing of the original skin lesions. *C. violaceum* was the only organism ever incriminated

during the course of his infection. Unfortunately no necropsy was performed through which further study could have been made.

In 1939 Soule, in Michigan recorded the second fatal instance of human infection in which *C. violaceum* was isolated. The necropsy protocol of this case was presented by Weller. Pure cultures were obtained from the heart's blood, spleen and liver of a girl of fifteen years. This patient for nine months prior to death had suffered from a cervical adenitis which was diagnosed clinically and by biopsy, as tuberculosis even though *C. violaceum* was isolated from "pus" aspirated from the lesion. The tuberculin test, X-ray of the chest, as well as special cultures and guinea pig inoculation revealed no evidence of tuberculous infection. The material for animal injection was as usual, first treated with 4% potassium hydroxide. This chemical treatment apparently killed any *C. violaceum* present and would account for its failure to infect the animal. At necropsy of the patient, focal necrotized lesions were found in the lungs, spleen, liver and pelvic peritoneum which grossly resembled tuberculosis. Histologically these lesions proved to be of a necrotized, proliferative and, to some extent, pyogenic type presenting no essential evidence of tuberculous reaction. *C. violaceum* was recovered from the heart's blood and "pyemic abscesses" in the liver. The strain of *C. violaceum* isolated in this case was compared by Soule with the culture of Black and Shahan and with "2 old laboratory strains" of *C. violaceum*. Each strain produced the characteristic deep violet color *in vitro*, and the 4 strains appeared to be identical morphologically. Certain cultural differences were, however, noted. With experimental animals, he found that broth suspensions of each of the 4 strains were toxic in quantities of 0.5 ml.

In our recently encountered instance, the infection of *C. violaceum* demonstrated a rapid and fulminating invasion of the human subject. The patient was a robust young colored man who previously had enjoyed excellent health. There was no history of protracted or obscure infection occurring over many months as in the other reported cases. A rapid and definite invasion by *C. violaceum* occurred through a thorn wound of the foot with resulting inguinal adenitis, septicemia (positive blood culture) and death of the patient seven days after the onset. The details of the clinical case including the summarized necropsy are reported elsewhere (Schattenberg 1941).

At necropsy cultures were prepared upon various types of media from the lesions of the liver, lungs, inguinal lymph nodes and heart's blood and all of these yielded heavy growths of *C. violaceum*.

SALIENT AND COMPARATIVE PATHOLOGY

A marked necrosis of the inguinal lymph glands and disseminated bizarre-shaped pulmonary and hepatic nodules represented the tangible gross pathological factors present in this case. From the microscopic standpoint, the outstanding characteristic feature consisted of necrosis with comparatively little cellular response (figs. 1 and 2).

The necrosis did not conform grossly to the curdy or granular aspect of caseation nor was it similar to the pathological aspect of coagulation necrosis. It

had in the gross, the firmness of the latter lesion but microscopically conformed to the former or tuberculous necrosis. *En masse*, however, as found in the extensive glandular lesions there was evidence of liquefaction necrosis.

The reported instances of human infection with *C. violaceum* by Black and Shahan, and Soule present a clinicopathological aspect different from the present case. In the report of Black and Shahan, while systemic symptoms were recorded, the external skin lesions were emphasized. Inguinal adenitis had occurred in the early onset and eventually, after some months, extensive cervical adenitis developed during what was considered a "flare up" or recrudescence leading to death. Unfortunately no necropsy was held and no bacteriological

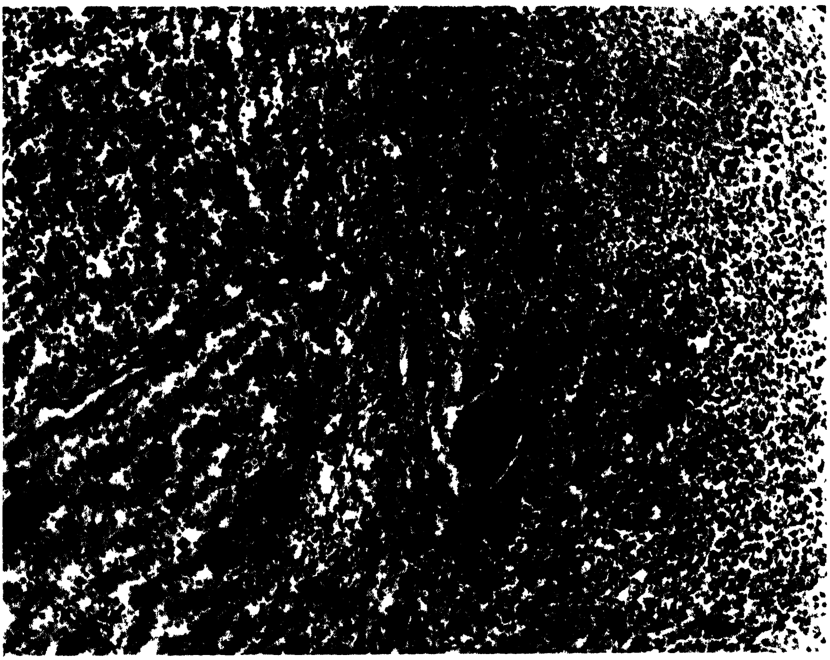

FIG. 1. PHOTOMICROGRAPH OF HUMAN INGUINAL LYMPH GLAND

Approximately two-thirds of the right portion demonstrates extensive necrosis of the lymphoid structure. × 150.

study during the terminal "relapse" was reported. The total period of time involved from infection to death was fifteen months. The report of Soule has as yet appeared only in abstract form. This case was also of protracted duration, lasting nine months. A massive cervical adenitis was considered as tuberculous although only *C. violaceum* had been recovered by aspiration from the lesion. While a biopsy indicated possible tuberculosis, all efforts to demonstrate *Mycobacterium tuberculosis* failed, including special culture and guinea pig inoculation. The validity of infection by *C. violaceum* appeared, however, to have been established through the necropsy, at which time this microörganism was recovered from the heart's blood, liver and lung lesions. The pathology of

the cervical glands represented both a chronic proliferative and pyogenic type of reaction. This work is to be published more fully at a later time after continued study and observations have been completed.

It is noteworthy that marked adenitis has been found in all recorded human cases. Woolley also noted in the carabao marked enlargement of the prescapular glands. The rapidly fatal manifestations observed in these animals suggested to him hemorrhagic septicemia of cattle. The description of the

FIG. 2. PHOTOMICROGRAPH OF A NECROTIC AREA IN THE HUMAN LUNG
Extensive tissue destruction and karyorrhexis with resultant confluence of structure is present. × 100.

lesions found at necropsy by Woolley conformed in general to those observed in our human subject. He obtained positive cultures of *C. violaceum* from the lymph nodes and lung lesions. His cultures proved pathogenic for many types of lower animals. He was convinced that *C. violaceum* was the causative factor of infection in the carabao. It may be stated that in general the clinical, bacteriological and pathological features of the human infection herein concerned conformed in all respects to those reported by Woolley in the carabao.

EXPERIMENTAL

Certain phases of experimental study have been undertaken by us with the culture of *C. violaceum* isolated from the human infection herein described. These consist of studies upon pathogenicity with reproduction of the disease in the lower animals, mouse protection against infection and experimentation appertaining to the probable route of infection. One of us (Schattenberg 1940–1941) has recently presented preliminary reports upon certain of these observations. It is intended herein to stress especially the salient observations upon the pathogenicity of this strain for experimental animals and to deal briefly with the experiments upon protection against infection as well as those bearing upon the probable route of invasion by *C. violaceum*.

Pathogenicity

In all, fifty white mice, twelve rabbits, and ten guinea pigs were utilized.

White mice. The cultures employed were principally subcultures of that recovered from the human host. These cultures were grown in glucose broth and on nutrient agar. The injections of suspensions containing approximately one billion microörganisms per ml. were given intra-abdominally. All injected animals died and *C. violaceum* could readily be recovered from the peritoneal cavity and heart's blood. Where gross lesions were evident in the lungs, liver and spleen, positive cultures were obtained therefrom. Death of these animals occurred in from ten hours to three days, according not only to dosage but the media upon which the culture was grown. It was noted that the broth cultures, even in smaller doses, proved more virulent than the washings of the solid slanted medium. The doses of each type of suspension varied from .05 ml. up to 1 ml. In those animals that survived for three days gross lesions were found which conformed to those described in the human case. Microscopically the pathological changes were likewise similar. In the animals overwhelmed within a few hours, no discernible lesions could be found with the naked eye and only slight parenchymatous degenerative change was noted microscopically in the liver, kidneys and spleen.

Rabbits. Twelve animals were employed, six receiving intravenous injections and six others, intra-abdominal injections. The rabbits given intravenous injections, received 1 ml. of a saline suspension of *C. violaceum* which was markedly chromogenic and had been grown on nutrient agar. The suspension was diluted to contain one billion microörganisms per ml. Following these injections all animals died within twenty-four hours. Necropsies showed no gross lesions. *C. violaceum*, however, was readily recovered by culture from the lungs, liver, spleen, and heart's blood. Microscopically, degenerative changes were noted in all organs, but particularly in the liver.

Six other animals were injected intra-abdominally with varying amounts of a *C. violaceum* broth culture. Increasing amounts of 0.5 ml., 0.75 ml., 1 ml., 1.5 ml., and 2 ml. were given respectively. The two rabbits receiving the larger amounts, i.e., 2 ml. and 1.5 ml. died in twelve and twenty-four hours respectively. *C. violaceum* was recovered by culture from the organs and heart's

blood. The two rabbits receiving 1.25 ml. and 1 ml. survived for five days. At necropsy the lungs (fig. 3), liver and spleen showed yellowish-white necrotized areas varying from 1 mm. to 4 mm. in diameter. On microscopic examination these foci showed a central necrosis of the parenchymatous cells with a peripheral zone in which the tissue cells showed degenerative changes. There was only a slight cellular response which consisted mainly of an occasional neutrophile, and a few lymphoid and plasma cells.

The lesions, both grossly and microscopically, bore a striking resemblance to those in the human infection. As in the human case studied by us, no violet color of the lesions was noted in the experimental disease. *C. violaceum* was, however, readily recoverable on culture from the organs and also the heart's blood and presented the rich violet chrome.

The two rabbits receiving 0.5 ml. and 0.75 ml. respectively have survived.

FIG. 3. LUNGS FROM RABBIT SURVIVING FOR FIVE DAYS SUBSEQUENT TO INTRAABDOMINAL INJECTION OF B. VIOLACEUS
Note subpleural nodules present in both lungs

Guinea pigs. A group of ten animals were injected intra-abdominally with suspensions of *C. violaceum*. The organism was grown on nutrient agar slants, washed off with sterile saline and the suspension diluted to contain 1 billion bacteria per ml. Five guinea pigs received 0.25, 0.5, 0.75, 1.0 and 1.5 ml. respectively of this suspension. Three of these animals died within twenty-four hours. Necropsy showed occasional minute white scattered areas in the liver on gross examination. The lungs appeared congested. Microscopically the liver cells were granular and necrotic, presenting fragmented nuclei. *C. violaceum* was cultured from the heart's blood and parenchymatous organs. Two remaining guinea pigs which received 0.25 and 0.5 ml. survived. Another group of five guinea pigs were given intra-abdominal injections of a broth culture of *C. violaceum* in similar amounts and also diluted to contain approximately 1 billion bacteria per ml. All of the animals in this group died within

sixteen hours. On histological examination the parenchymatous organs showed toxic degenerative changes, especially in the liver. The hepatic lesions showed swollen, granular changes in liver cells with occasional foci which presented areas of marked necrosis. *C. violaceum* was recovered from the heart's blood and organs.

Route of infection

Because of the history of a splinter wound in our human case and the probable invasion through surface injury with adenitis in one other reported human case as well in the infected carabao or water-buffalo, it was considered that skin surface abrasion might have been the portal of entry of infection. Accordingly, experiments were carried out with rabbits and white mice wherein surface injury was produced and liability of infection with *C. violaceum* rendered likely.

In five rabbits, the fur of one hind paw was clipped and an incision approximately 1 cm. in length was made through the skin and into the subcutaneous tissues. Splinters consisting of small portions of tooth-picks measuring approximately 6 mm. in length were dipped in a suspension of living *C. violaceum* and inserted in one end of this small incision. Three additional rabbits, after clipping the fur on the foot, received small crucial incisions down to the subcutaneous structure. The wounds were then swabbed with a broth culture of *C. violaceum*. Oozing of fluid was present in the fresh wound, hence on the following day, the crust was removed and a similar swabbing carried out.

The five rabbits receiving the infected splinters showed swelling and retraction of the infected limb within twenty-four hours and became definitely ill. One died in four days and two others in eight and nine days respectively. The two remaining rabbits survived.

The necropsies of these animals all revealed pathological evidences of severe *C. violaceum* infection, lesions being present in the heart, lung and liver (fig. 4). Congestion and swelling of lymph nodes was present along the path of invasion especially for those animals surviving for the longer periods. The gross and microscopic study was similar to that already described. *C. violaceum* was recovered in culture from the affected organs and the heart's blood of all animals.

Of the three rabbits wherein crucial incisions were made and exposure to living *C. violaceum* suspension had occurred, two showed swelling and oedema of the infected foot and leg. One died in eleven days and another in two weeks. The remaining animal survived. The necropsy findings in these animals were similar in all regards to those of the rabbits receiving splinters immersed in the *C. violaceum* suspensions. Four control rabbits wherein the fur was clipped but no abrasions of the paws were present, were swabbed in this area with a suspension of living *C. violaceum* but remained unaffected.

Twenty white mice were also submitted to *C. violaceum* infection. In ten of these, one of the hind foot-pads was scarified to the subcutaneous structure. The feet of these ten together with the feet of the ten control mice were dipped into or swabbed with a dilute suspension of living *C. violaceum* for a period of a minute on two successive days. Of the ten mice in which scarified wounds

had been produced, eight were preceptibly sick in four to six days. In four of these animals wherein death was impending, cultures were made from the heart's blood and yielded heavy growths of the chromogenic bacillus. Four others died twenty-four to forty-eight hours later. Two mice remained normal except for some temporary impairment of function in the affected leg. The infected animals, as in the instance of the rabbits, showed swelling and retraction of the leg wherein infection had occurred. All of the control mice remained normal.

The necropsies of the infected mice all revealed the same bacteriological and pathological findings as for the infected rabbits although several of these mice showed unusual multiple seeding of very small lesions, particularly in the liver.

Fig. 4. Liver of Rabbit that Died Nine Days Subsequent to Introduction of Splinter Infected with B violaceus
Necrotic foci of varying sizes scattered beneath Glisson's capsule

It is evident from this series of experiments that *C. violaceum* may readily gain access to the host, causing infection and death, by route of body surface injury.

Protection against infection

In white mice that had been tested for the toxicity of Seitz filtrates of broth cultures with negative results, it was noted that when these were grouped with normal animals and injected with living culture they remained unaffected, whereas the normal animals died. Accordingly, tests were carried out to ascertain the constancy of such observations. Twenty white mice received two

intra-abdominal injections of 0.5 and 1.5 ml. of Seitz filtrate from broth cultures of *C. violaceum* grown for twenty-four and ninety-six hours apart. Two days subsequent to the last injection, all animals were injected intraäbdominally with 0.1 ml. of a suspension of living *C. violaceum.* Twenty normal mice were used as controls for the pathogenicity of the same living suspensions, 10 receiving 0.1 ml. and 10 .05 ml. intra-abdominally. The protection afforded was complete, in that the twenty mice previously injected with broth culture filtrate survived whereas all controls died within three days. Three weeks of observation without apparent illness was considered as a survival period. In order to ascertain if the protein of the media may have occasioned reactions and thereby blocked invasion, five white mice were administered sterile nutrient bouillon intra-abdominally. This was given in the same doses and time intervals as for the broth culture filtrate and similarly 0.1 ml. of the living *C. violaceum* suspension was injected two days later. All of these animals subsequently died in from 24 to 72 hours.

It is apparent that definite protection against a lethal dose of *C. violaceum* is afforded by the broth culture filtrates. We have not as yet determined the matter of its specificity. It is evident that the bouillon also did not occasion an inflammatory protective reaction. Further work is in progress upon this problem relative to the specificity or simple local reaction affording protection.

COMMENT

It is noteworthy in the infection by *C. violaceum* presented herein as well as those observed in the carabao or water-buffalo, as reported by Woolley, that the infection was rapid and fulminating in its clinical course, proving fatal in a brief period of time.

In our case and probably those of the carabao, infection of the feet and involvement of drainage lymph nodes represented the mode of invasion. Woolley refers to the adenitis present in the prescapular glands. The reports of Black and Shahan and of Soule show, on the other hand, a very protracted illness with an enduring cervical adenitis and ensuing death after many months duration. In Black and Shahan's report, however, there was a history of primary inguinal adenitis. It appears rather remarkable that only these few authentic infections with this supposedly non-pathogenic microörganism have been recorded and furthermore that all have resulted in fatality. Black and Shahan's report presents the only notation of manifestation of a violet pigment in the lesions produced.

Bacteriological considerations

It has heretofore been generally represented that *C. violaceum* is rather ubiquitous in its distribution. This fact is recognized in certain geographical areas as regards the field of general bacteriology. We are informed by Dr. A. T. Henrici that those working extensively with natural waters are well acquainted with this group of microörganisms. He estimates that violet

colonies have appeared on plates made from about one out of twenty samples of lake water. In our experience and those here locally with whom we have conferred, its occurrence, even as a contaminant, has not been observed in medical bacteriological routine examination. In regard to food-stuffs, one of the members of the Federal Pure Food Division informs us that in an experience of five years in the examination of foods, often of contaminated character, he has never encountered this group of microörganisms. Even the variants of *Chromobacterium violaceum* that have been described, appear as of rare medical bacteriological occurrence. In this connection we have communicated with investigators in medical bacteriology in various sections of the United States as to their observations upon this group. The responses, all of negative character, are as follows: Parker at Boston has never encountered these microorganisms in routine bacteriological laboratory examinations amounting to approximately 20,000 per annum. Feemster also at Boston reports no personal contact. He states that others in the Public Health Division have seen violet colonies in the examination of water supplies. Bayne-Jones at New Haven, Turner at Baltimore, Hektoen and Cannon in Chicago, Meyer at San Francisco and Bohls at Austin, all report negative experience for *C. violaceum*. As this information is from representative men of experience who are located in widely distributed areas, it appears fully justifiable to state that this microörganism is encountered very rarely in the field of medical bacteriology in this country.

Because of the rich purple and violaceous chromes produced by these microorganisms, they must necessarily attract attention in examination of bacteriological plantings or of cultures made in routine examination. In addition to this fact this group grows very readily upon ordinary culture media. Because of these two features it is evident that any widespread distribution would necessarily be noted by those working in the field of medical bacteriology.

While *C. violaceum*, without differentiation, is generally considered saprophytic and non-pathogenic, it appears evident that in those instances wherein the pathogenic type has been isolated as the infectious agent, it has proven to be fatal to the human subject and consistently and promptly pathogenic in its effect upon the ordinary laboratory animals. We have observed that this property of pathogenicity remains constant although the microörganism has been perpetuated by sub-cultures for many months. Thus, our own strain now in continuous cultivation for over five months and likewise the strain of Black and Shahan received in this laboratory more than two years ago, still demonstrate strong pathogenicity for laboratory animals. Certainly there is no sudden reversion to an innocuous character manifested by these cultures. There seems but little doubt that the general statements regarding absence of pathogenicity have been applied to *C. violaceum* considered as one microörganism without recognition of distinctive types. It has not been universally appreciated that while members of this group show relationship in color, they differ distinctly in cultural activities and inoculation effects upon animals.

Although highly pathogenic for lower animals, the Seitz filtrate obtained from broth cultures of the present strain, grown for a period of a week or more,

fail to reveal any potent action upon animals. It is, however, noteworthy in this regard that white mice receiving two injections of such filtrates are immune to injection of the living bacilli in dosage even twice as large as those killing control animals within twenty-four hours.

It has been shown herein through animal experimentation, that surface injuries form plausible atria and routes of infection and invasion with *C. violaceum.*

Pathology

The gross pathology of both the human being and the carabao wherein fulminating infection and death have occurred reveals a rather consistent picture. This is evidenced by nodular lesions varying from minute foci to those as large as 1 cm. in the longest diameter. They may be rounded or oval in shape but are usually of bizarre or irregular outline. Their color is generally a dirty white. They are of firm consistency although appearing somewhat granular upon cut surface. They are present more especially in the lungs, liver and spleen but may occasionally occur in other structures. When the drainage lymph nodes are involved, massive destruction and liquefaction may occur. Microscopically, the changes represent a granular necrosis of structure suggestive of escharotic action. The parenchymatous units, naturally, show more severe changes than does the framework tissue. There appears but little cellular or other exudative response on the part of the host although occasionally some lymphoid or polymorphonuclear neutrophilic cells may be present peripherally to the essential necrotized lesion. The experimentally produced lesions of the various animals employed reveal a distinct duplication of the natural disease both from the gross and microscopic study.

Comparative considerations involved

The varying clinical manifestations of infection in the human subject together with the nonspecific gross and microscopic pathological picture although consistent in character, suggest the likelihood that other such occurrences may have passed unrecognized. The clinical findings are quite similar to those presented in certain other disease entities such as fatal tularemia and *Pasteurella pseudotuberculosis* or rodentium infection. In this group, aside from somewhat similar clinical manifestations, the gross pathological findings consists of localized areas of greyish-white nodules in the lung, liver and spleen. An accurate differential diagnosis of these closely allied conditions depends obviously upon isolation of the etiological agent. Identification of *C. violaceum* is most simple because of the facility of its cultivation and characteristic rich violet pigment production upon the ordinary types of laboratory media. It is not unlikely that because of the simplicity of culture and the general acceptance of *C. violaceum* as a harmless pigment-producing saprophyte, its isolation in some instances has been regarded as a contaminant rather than as the true etiological factor which it represented. It is to be also noted that the simple finding of gram-negative bacilli in smears of lesions as well as uncertain agglutination reactions may form misleading factors of diagnosis in this allied group of diseases.

SUMMARY

1. A fatal infection of a human subject with *Chromobacterium violaceum*, var. *manilae*, formed the basis for these observations.

2. Two particular experimental observations have been made. First, the demonstration of definite pathogenicity and the reproduction of the disease in the lower animals and second, the protection afforded against experimental infection with *C. violaceum* by broth culture filtrates. In addition, it has been shown herein that fatal infection with this microörganism may be induced in animals through experimentally produced wounds of the feet, thus indicating a likely route of invasion in the natural infection of man or animal.

3. Emphasis is laid upon the fact that, in at least medical bacteriology routine, *C. violaceum* is not widespread in its occurrence in this country. Serious question is also raised as to the wisdom of regarding such cultures as innocuous or non-pathogenic saprophytes.

4. It is stressed that *C. violaceum* has been in general, erroneously considered only as a single species. In reality, it is one of a group wherein cultural and biological methods permit of definite differentiation. There seems but little doubt that many of the other members of this group are truly non-pathogenic saprophytes.

5. The culture concerned in the present observations conforms to that described by Woolley and identified by him as *C. violaceum*, var. *manilae*.

REFERENCES

Bergonzini, Curzio 1881 Sopra un nuova Batterio Colorato. Annûario della Societa dei Naturalisti in Moderna, Ser. 2, **14**, 149–158.

Black, M. E., and Shahan, J. 1938 *Bacillus violaceus infection* in a human being. J. Am. Med. Assoc., **110**, 1270–1271.

Ford, William W. 1927 Textbook of Bacteriology. W. B. Saunders Company, Philadelphia and London, 468–475.

Schattenberg, H. J., and Harris, W. H. 1941 A definite and unique occurrence of rapidly fatal infection caused by *Bacillus violaceus* Manilae. J. Am. Med. Assoc., **117**, 2060–2070.

Schattenberg, H. J. 1940 Experimental studies upon *B. violaceus* isolated from a fulminating infection. Proc. Soc. Exptl. Biol. Med., **45**, 829–831.

Schattenberg, H. J., and Brown, D. V. 1941 Experimentally produced wounds as a route of fatal infection caused by *Bacillus violaceus*. Proc. Soc. Exptl. Biol. Med., **46**, 478–482.

Soule, M. H. 1939 A study of two strains of *B. violaceus* isolated from human beings. Scientific Proc. Am. Assoc. Pathologists and Bacteriologists, Am. J. Path., **15**, 592–595.

Woolley, P. G. 1905 *Bacillus violaceus* Manilae. (A pathogenic microörganism.) Bull. Johns Hopkins Hosp., **16**, 89–93.

THE DEHYDROGENATION OF ALCOHOLS BY STREPTOCOCCI OF GROUP B

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The most characteristic metabolic activity of streptococci is the fermentation of carbohydrates to lactic acid. Although this process accounts almost quantitatively for the carbohydrate fermented, Friedemann (1938, 1939) has shown that traces of ethyl alcohol and volatile acids occur when growing cultures ferment glucose, thus indicating that the predominant metabolic process of these organisms is not their only fermentative process.

Using glucose as a substrate for dehydrogenation studies with methylene blue as hydrogen acceptor, Wood and Gunsalus (1942) have established conditions for the production of resting cell suspensions of streptococci with strong dehydrogenase activity toward this substrate. In a search for other substrates which could be activated as hydrogen donors it was found that ethyl alcohol was even more active than glucose, while organic acids were inactive or very weak hydrogen donors. This result is opposite to that found by Quastel and Whetham (1925) in their studies of *Escherichia coli*. Although *Escherichia coli* produces considerable quantities of ethyl alcohol among its fermentation products, it has only very weak dehydrogenase activity toward alcohols.

This strong dehydrogenase activity of streptococci for ethyl alcohol led us to make the following study of alcohols in general with group B streptococci.

EXPERIMENTAL

Five strains of group B streptococci from the departmental culture collection were used. Three strains (Nos. 68c, 70b, and 83a) were originally isolated from milk. All had originally fermented lactose but at the time these experiments were performed two (68c and 70b) had lost the power of fermenting lactose. Two strains (Nos. G1, 44P1) were from human sources; 44P1 isolated from human feces, and G1 from a kidney infection. Strain G1 did not ferment lactose when isolated. All strains were members of Lancefield's group B hemolytic streptococci and had the usual physiological characteristics of this group of organisms (Sherman 1937). One strain of *Escherichia coli* (71) was carried in the experiments for comparative purposes.

The cell suspensions were obtained as suggested by Wood and Gunsalus (1942). Cells grown 12 hours at 37°C. in a 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent K_2HPO_4, 0.1 per cent glucose medium were collected by centrifugation, washed once with $\frac{1}{2}$ growth volume of M/30 phosphate buffer, pH 7.4, and resuspended in $\frac{1}{10}$ growth volume of the same buffer. The ability of the organism to activate various substrates as hydrogen donors was deter-

mined by observing the reduction of methylene blue in Thunberg tubes containing the following:

In side arm: 1 ml. 1:4000 methylene blue.

In tube: 2 ml. M/15 phosphate buffer pH 7.2, 1 ml. substrate (glucose M/20, alcohols 1%), 1 ml. cell suspension in M/30 phosphate buffer.

After evacuation, the tubes were allowed to come to the temperature of the bath, the methylene blue tipped into the tube, and the time required for 90 per cent reduction recorded.

It should, of course, be realized that not all compounds activated by organisms need donate hydrogen to methylene blue under these conditions. Positive data so obtained, however, are useful. All the alcohols employed as substrates were Eastman Kodak products used without further purification; appreciable quantities of impurities in them would lead to erroneous results as only a very small

TABLE 1

Effect of substrate concentration on reduction rate

S. mastitidis (70b), (0.23 mg. bacterial nitrogen/tube)·7.2 40°C

PER CENT ALCOHOL	MINUTES FOR 90% REDUCTION OF 1:20,000 METHYLENE BLUE		
	Ethyl	Isopropyl	Propyl
20	42	>60	
15	6		
10	2.4		>60
8	2.0		2
5	2.0	1.3	1.5
2	2.0	1.2	1.5
1	2.0		1.5
0.5	2.0	1.2	1.5
0.1	3.2	1.3	1.5
0.05	5.4		2.8
0.02	9.4	2.5	4.5

amount of methylene blue is present (1.6×10^{-4} molar). Quantitative methods to determine the products formed would be indicated once it is known that a compound is activated by the organisms.

Substrate concentration

With glucose as substrate a final concentration of M/100 in the tube proved satisfactory. With alcohols any concentration added would be decreased by volatilization during evacuation, and, on the other hand, too high a concentration would be inhibitory. Table 1 indicates the range within which the concentration of alcohol is not a factor in the reduction rate. As can be seen from the table, propyl and isopropyl alcohols are toxic at lower concentrations than is ethyl. However, any concentration from 0.5 to 5 per cent is satisfactory for these alcohols. On the basis of these data a concentration of 1 per cent alcohol (0.1–0.2 molar) was selected as satisfactory for dehydrogenase studies with

these streptococci. Quastel and Whetham (1925) found the inhibitive action of alcohols toward dehydrogenase systems of *Escherichia coli* increasing with the length of the carbon chain. In their studies the lactic and succinic dehydrogenase systems were inhibited by ethyl alcohol at about 25 per cent and propyl at about 10 per cent concentration.

pH optimum

In order to compare the rates of dehydrogenation of alcohols with that of glucose it would be desirable to work as near to the optimum of each as possible. As can be seen from table 2, the optimum range for glucose is slightly more acid than the optimum for the alcohols. A fairly broad optimum, half of a pH unit, exists for glucose and an even broader optimum for the alcohols. In order not to decrease the rate for glucose and still work near the optimum for the alcohols, a pH of 7.2 was used in subsequent experiments.

TABLE 2

Effect of pH on rate of dehydrogenation

S. mastitidis (70b), (0.22 mg. bacterial nitrogen/tube.) 40°C

pH IN THURBURG	MINUTES FOR 90% REDUCTION OF 1:20,000 METHYLENE BLUE		
	M/100 glucose	2% ethyl alcohol	2% isopropyl alcohol
5.8	5.5		
6.1	4.5	3.5	2.1
6.4	3.5	3.0	1.8
6.8	3.3	2.5	1.5
7.1	3.2	2.1	1.2
7.3*	3.1	1.9	1.2
7.7	7.5	2.0	2.3
8.0	11.3	3.3	3.5

Alcohols dehydrogenated

In order to determine whether other alcohols were dehydrogenated by these streptococci 5 strains were tested on a number of alcohols and glycols. The rates of dehydrogenation of the various alcohols, based on the time required for 90 per cent reduction of 1:20,000 methylene blue compared to the rate with glucose taken as 100, are given in table 3. Methyl alcohol was not activated by any of the streptococci, nor by *Escherichia coli*. The other normal alcohols from ethyl to amyl were all activated and were equal to, or more active than, glucose as hydrogen donors. Propyl alcohol was the most active of the normal alcohols, although only slightly more active than ethyl. Butyl and amyl alcohols ranged from activity equal to ethyl (two cases) to about half the activity of ethyl, or approximately equal to that of glucose. Among the secondary and tertiary alcohols, isopropyl, secondary butyl, and tertiary amyl were found to be very active hydrogen donors under the conditions studied. In fact, all three were more active than the normal alcohols, the activity ranging up to 6 times that of glucose in the case of isopropyl and secondary butyl. The high reactivity

of tertiary amyl is rather surprising. In view of the lack of hydrogen in the carbon atom to which the hydroxyl group is linked, it is difficult to write a reaction for the dehydrogenation, as an aldehyde or ketone could not be formed without breaking the carbon chain. Quantitative work with this substrate should yield interesting results. The oxidation products of the two highly-reactive secondary alcohols, isopropyl and secondary butyl, would not be as difficult to predict as would the tertiary amyl. The next secondary alcohol, 2-pentanol, while having the same spacial arrangement of the alcohol group as

TABLE 3

Dehydrogenation of various alcohols by S. mastitidis

(Rates compared to glucose = 100)

CULTURE	70b	68c	83a	44P1	G1	E. coli 71
GLUCOSE MINUTES	4.5	2.75	11.0	3.0	3.25	0.50
Glucose	100	100	100	100	100	100
Control	<2	<2	<2	<2	<2	<1
Alcohols:						
Methyl	<2	<2	<2	<2	<2	<1
Ethyl	140	170	130	200	185	2.2
Propyl	165	220	200	240	200	2.0
Butyl	80	180	105	150	90	2.2
Amyl	110	170	90	230	70	<1
Iso-propyl	260	240	625	400	220	1.4
Sec. butyl	225	275	625	225	220	1.5
2-Pentanol	110	110		50	40	1.5
Methyl-isopropyl carbinol	30	60	40	4	10	
Iso-butyl	11	20	30	30	18	<1
Tert. butyl	10	<5	<5	<2	<2	<1
Tert. amyl	225	275	290	120	130	1.4
Glycols:						
Ethylene	<2	<2	<5	<2	<2	10
Trimethylene	180	45	25	65	20	9
2,3 butylene	53	220	730	330	190	5
Gylcerol	<2	7	<5	<2	<2	10
Mannitol	<2	<2	<5	<2	<2	25

the 3 and 4 carbon secondary alcohols, has only about half the reactivity under the conditions used. Its reaction rate, however, is still equal to that of glucose. The other butyl and amyl alcohols tried were less active, ranging from half the activity of glucose with methyl isopropyl carbinol to no activity with tertiary butyl. *Escherichia coli* had only slight activity toward the alcohols.

Among the poly-hydroxy alcohols these streptococci did not activate ethylene glycol nor mannitol, as hydrogen donors. The latter result agrees with fermentation data, mannitol not being fermented by group B streptococci. Tri-

methylene glycol was active as a hydrogen donor as was 2,3 butylene glycol. With 2 of the strains of streptococci the latter compound was the most reactive of those studied. There is similarity in structure between 2,3 butylene glycol and the other secondary alcohols which were highly reactive, namely, isopropyl and secondary butyl. In contrast to the glycols, glycerol was only slightly reactive as a hydrogen donor toward one strain of group B streptococci. That this is not a true picture of the ability of the organism to attack glycerol will be discussed later under adaptation. *Escherichia coli* attacked mannitol as well as glycerol and the glycols. These reactions were very slow in comparison to rates with glucose and organic acids as hydrogen donors. The results with *Escherichia coli* are in agreement with those found by Quastel and Whetham (1925).

Adaptation to substrates

If the organisms to be used for the preparation of resting cell suspensions are grown in the presence of the substrates upon which they are to be tested instead of on glucose, it should be possible to study the formation of adaptive enzymes

TABLE 4

Formation of adaptive enzymes for galactose

Streptococcus mastitidis (68c) pH 7.2, 40°C. 90% reduction 1/20,000 methylene blue

GROWN ON	MG. BACT. N/ML. OF SUSPENSION	REDUCTION TIME IN MINUTES ON			
		Glucose	Fructose	Mannose	Galactose
Glucose	0.24	3.8	3.8	3.8	45.0
Fructose	0.22	5.0	4.5	6.5	43.0
Mannose	0.21	3.2	2.8	3.0	50.0
Galactose	0.24	2.5	2.0	2.8	2.

by the methylene blue technique. With sugars which serve as readily-available energy sources for streptococci, the method is satisfactory as indicated by the example in table 4. Growing the culture in galactose broth increases the rate of dehydrogenation on galactose by about 25 times over the rate when the cells are grown in the presence of glucose, fructose, or mannose. The rate of dehydrogenation on the other hexoses does not appear to depend upon the growth substrate, i.e., the catalysts are constitutive enzymes (Dubos, 1940). The adaptation of this strain to galactose is analogous to the results of Hegarty (1939) with *Streptococcus lactis*. All of the cultures of *Streptococcus mastitidis* do not show so great adaptation to galactose as does the 68c strain.

The results obtained with glycerol, which is not reported to be fermented by these organisms, are more complex. As recorded in table 3, when grown in glucose only one of the 5 strains of *Streptococcus mastitidis* was capable of dehydrogenating glycerol, and this one slowly. After the cultures had been transferred serially upon glycerol broth, all dehydrogenated glycerol, some at a rate approaching that for glucose. At first this appeared to be an adaptation of the

type reported above for galactose, but further experiments have shown that factors other than cultivation in the presence of glycerol affect the dehydrogenation of this compound with methylene blue as hydrogen acceptor. Some streptococci dehydrogenate glycerol rapidly when harvested from a growth medium to which no glycerol has been added.

Barron and Jacobs (1938) have reported that certain hemolytic streptococci of human origin oxidize glycerol in manometric experiments with oxygen as the hydrogen acceptor instead of methylene blue. They indicate that the rate of glycerol oxidation is variable but do not relate it to adaptive enzyme formation. These authors also report that those cultures which oxidize glycerol oxidize ethyl, propyl and butyl alcohols in manometric experiments.

SUMMARY

Resting cell suspensions of *Streptococcus mastitidis*, Lancefield group B, have been found to oxidize a number of alcohols, as indicated by methylene blue reduction in Thunberg tubes. Several of the alcohols, including the normal alcohols C_2 to C_5, secondary alcohols from C_3 to C_5, tertiary amyl alcohol, and 2,3 butylene glycol are more active as hydrogen donors to methylene blue than is glucose. Methyl alcohol is not activated by these organisms. Glycerol is dehydrogenated rapidly by some strains, but the factors contributing to its dehydrogenation are not completely elucidated. An adaptive mechanism is present in some strains for the dehydrogenation of certain sugars, as for example, galactose.

A strain of *Escherichia coli*, studied for comparative purposes, was found to dehydrogenate alcohols very slowly. This observation is in agreement with the results of Quastel and Whetham.

REFERENCES

Barron, E. S. G., and Jacobs, H. R. 1938 Oxidations produced by hemolytic streptococci. J. Bact., **36**, 433–449.

Dubos, R. J. 1940 The adaptive production of enzymes by bacteria. Bact. Revs., **4**, 1–16.

Friedemann, T. E. 1938 The metabolism of pathogenic bacteria. I. Bacteriological and chemical methods. J. Bact., **35**, 527–546.

Friedemann, T. E. 1939 The carbohydrate metabolism of Streptococci. J. Biol. Chem., **130**, 757–761.

Hegarty, C. P. 1939 Physiological youth as an important factor in adaptive enzyme formation. J. Bact., **37**, 145–152.

Sherman, J. M. 1937 The Streptococci. Bact. Revs., **1**, 1–97.

Quastel, J. H., and Whetham, M. D. 1925 Dehydrogenations produced by resting bacteria. I. Biochem. J., **19**, 520–531.

Wood, A. J., and Gunsalus, I. C. 1942 The production of active resting cells of streptococci. J. Bact., **44**, 333–341.

CELL INCLUSIONS OF GLOBIFORME AND RELATED TYPES OF SOIL MICROORGANISMS

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Many soil microorganisms are known to produce cell inclusions, and the demonstration of such bodies in any microbial group does not imply particular cytological or taxonomic distinction for that group. Recognition and identification of cell inclusions does, however, encourage the correct interpretation of cytological appearances or of variations therein (Lewis, 1937, 1938). The globiforme and related types of soil microorganisms have remained a morphologically puzzling group, which nevertheless has become recognized as a group culturally predominant in many soils.

Conn, after reporting (1925) the occurrence of considerable numbers of coccus-forming rods in soil, described (1928) *Bacterium globiforme* as the predominant if not the sole species accounting for the globoid rods abundant in certain productive soils. Later, Taylor and Lochhead (1937) reported globiforme rods obtainable from soils of either low or high fertility. The common distribution of *B. globiforme* and diphtheroid rods in soil has been widely confirmed (Jensen, 1933; Topping, 1937; Lochhead, 1940; Clark, 1940).

The characterization and grouping of a considerable number of globiforme isolates from soil, and their comparison with culturally similar types from other laboratories, has required their cytological study. It is the purpose of this paper to report briefly the nature of inclusion bodies present in *B. globiforme* and closely related microorganisms, and to discuss the possible role of inclusion bodies in the cellular pleomorphism and coccus-form development exhibited by this group.

EXPERIMENTAL

The following have been employed for cytological study:

Bacterium globiforme, 12 cultures. (1) American Type Culture Collection, no. 4336; (2), from N. R. Smith; (3–6), from A. G. Lochhead; (7–12), our isolates from soil.

Soil corynebacteria and types possibly related, 18 cultures. (13) *Corynebacterium simplex*, American Type Culture Collection, no. 6946; (14) *Corynebacterium tumescens*, American Type Culture Collection, no. 6947; (15) *Bacterium fimi* (*Corynebacterium fimi* Jensen; *Cellulomonas fimi* Bergey *et al.*), type culture from N. R. Smith.

(16–25), our isolates from soil.

(26–30), *Corynebacterium xerose*, *C. ovis*, *C. ulcerans*, *C. hofmanni*, and *C. hoagii*, from Julia M. Coffey.

Proactinomyces spp., 6 cultures (31–36), isolated from soil.

These microorganisms have been cultured on yeast-extract, nutrient, and asparagin agars, as well as upon such media with either glucose or glycerol added.

Subcultures were incubated at 28°C., and microscopic examinations were made both by fixed smear and by flat wet-mount procedures.

Staining methods. After comparison of numerous volutin-staining procedures, the following modification of methylene-blue-staining was found generally satisfactory. Films fixed with gentle heat or alcohol were exposed to the following solutions for the periods indicated, with light washing in distilled water following use of each different solution:

(1) Loeffler's methylene-blue solution, for 10 to 15 minutes;
(2) one-percent-sulfuric-acid, for 5 to 10 seconds;
(3) Lugol's iodine solution, for 15 to 30 seconds;
(4) aqueous safranine or aqueous fuchsin, for 2 to 3 minutes.

With this procedure, volutin bodies stood out sharply as dark or black granules in contrast to a light red cell background; there was a minimum of precipitate remaining on the slide. The dependability of the method was checked on *C. xerose*, *Lactobacillus helveticum*, and *Azotobacter*.

For staining fat inclusions, the ethylene glycol solution of Sudan Black B (Hartman, 1940) in flat wet-mount procedure, and the naphthol-blue method of Dietrich and Liebermeister (1902), have been found satisfactory. In flat wet-mount procedures for either fat or volutin, the occurrence of angular or branched cells, not uncommon in this group, coupled with Brownian movement, may produce out-of-focus and therefore darker-appearing cell portions; care must be taken to distinguish such portions from truly stained bodies. Also, because of the comparatively small size of globoid microorganisms, some differential staining procedures for volutin and fat readily applicable to such larger microorganisms as *Saccharomyces*, *Azotobacter*, *Spirillum*, *Rhizobium* or *Bacillus* may give results difficult of interpretation.

Volutin inclusions. During the early hours of growth, all cultures studied usually stained uniformly with aqueous aniline dyes, although occasionally broad diffuse bandings were observed. Irregular and globoid cells developed within one or two days, depending upon the culture and the substrate, and cell inclusions appeared. Certain of these could be considered volutin because of their characteristic dye affinities, solubility in hot water, and resistance to dilute acid de-staining. Volutin demonstrations (fig. 1) for *C. tumescens*, *B. fimi*, and many of the soil isolates were comparable to those obtained for *C. xerose* and other recognized diphtheroids. The appearance of volutin in *B. globiforme* cultures were somewhat less striking. Observations secured suggest that the dark-staining bodies in 2-week cultures of *B. globiforme* encountered by Taylor (1938, fig. 7) were probably volutin. Similar pictures of dark-staining bodies in *B. globiforme* were obtained following volutin-staining procedures. Although two or more volutin bodies could usually be observed in single cells of *Proactinomyces* cultures and in soil corynebacteria, single cells of *B. globiforme* showed, usually one, and seldom more than two volutin inclusions.

Fat inclusions. Although *B. globiforme* and typical diphtheroid rods from soil did not contain fat, the total absence of fat inclusions was not shown by all

coccus-forming rods. The individual cells of certain soil isolates contained, in addition to volutin, bodies which were not stained by aniline dyes, but which were stained intensely by the naphthol-blue and sudan-black methods. All cultures which produced both fat and volutin inclusions were believed to be *Proactinomyces*, as a distinctive early mycelium could be observed by direct agar microscopy. In such microorganisms, the appearance of fat bodies was variable. Longer rods from young cultures contained as many as three to six distinct fat bodies, and like numbers of volutin inclusions. Older globoid cells, particularly those grown on glycerol media, were inflated or distorted by one large fat body, and showed very little cytoplasm or volutin. Not all of the *Proactinomyces*, or mycelium-forming types, produced fat bodies, as two cultures (nos. 35, 36) of this group were fat-negative, although volutin-positive.

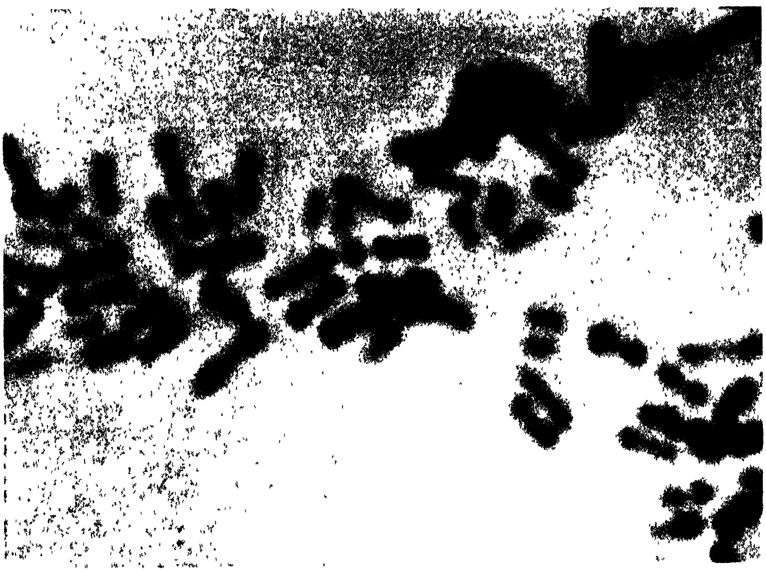

FIG. 1. PREPARATION OF SOIL ISOLATE NO. 21 SHOWING DARK STAINING VOLUTIN INCLUSIONS AND LIGHTLY STAINED CYTOPLASM. × 3840

Accordingly, it was impossible to separate *B. globiforme*, soil corynebacteria, and *Proactinomyces* cultures into these respective groups on the basis of cell inclusions.

Cell inclusions in relation to apparent cell morphology:

Conn (1928) and subsequent workers have noted that globiforme and related types of soil microorganisms appear as gram-negative rods in young cultures, and as gram-positive cocci in older cultures. In the present study, young cells have been noted to be gram-negative and rod-shaped, and to lack volutin inclusions. As the cultures increased in age, they passed through stages of gram-variability until they reached a gram-positive stage, appearing then either as volutin-filled coccus forms with short cytoplasmic "tails," or as short rods consisting primarily of two spherical volutin bodies, with little or no intermediate

cytoplasm apparent. Such cultures, when examined in the usual fixed-smear procedures, appeared to consist predominantly of cocci. That this appearance was in part illusory was shown by making parallel positively stained and negatively stained fixed smears from a uniform suspension of a single subculture; the former preparation revealed apparently coccoid, and the latter, distinct short rod, forms. The development of volutin bodies appeared to play an important role in determining cellular appearances. Taylor and Lochhead (1937) have previously observed that the characteristic globiforme transition is not merely a shortening from a rod to a coccoid stage, but that more complex and interesting phenomena are involved.

SUMMARY

The occurrence of volutin, and at times of fat inclusions, in coccus-forming rods abundant in soil, and techniques employed for their demonstration, are reported. It is suggested that volutin inclusions may play an important role in the cellular or globiforme transitions characteristic of microorganisms of this type.

REFERENCES

CLARK, F. E. 1940 Notes on types of bacteria associated with plant roots. Trans. Kansas Acad. Sci., **43**, 75-84.

CONN, H. J. 1925 Soil flora studies. VI. The punctiform-colony forming bacteria in soil. New York State Agr. Exp. Sta. Tech. Bull., no. 115.

CONN, H. J. 1928 A type of bacteria abundant in productive soils, but apparently lacking in certain soils of low productivity. New York State Agr. Exp. Sta. Tech. Bull., 138.

DIETRICH, A., AND LIEBERMEISTER, G. 1902 Sauerstoffubertragende Kornchen in Milzbrandbacillen. Zent. Bakt. Parasitenk, I., Orig., **32**, 858-865.

HARTMAN, T. L. 1940 The use of Sudan Black B as a bacterial fat stain. Stain Tech., **15**, 23-28.

JENSEN, H. L. 1933 Corynebacteria as an important group of soil microorganisms. Proc. Linnean Soc. N. S. Wales, **58**, 181-185.

LEWIS, I. M. 1937 Cell inclusions and the life cycle of Azotobacter. J. Bact., **34**, 191-205.

LEWIS, I. M. 1938 Cell inclusions and the life cycle of Rhizobia. J Bact., **35**, 573-587.

LOCHHEAD, A. G. 1940 Qualitative studies of soil microorganisms. III Influence of plant growth on the character of the bacterial flora. Can. J. Research, C, **18**, 42-53.

TAYLOR, C. B., AND LOCHHEAD, A. G. 1937 A study of *Bacterium globiforme* Conn in soils differing in fertility. Can. J. Research, C, **15**, 340-347.

TAYLOR, C. B. 1938 Further studies of *Bacterium globiforme* and the incidence of this type of organism in Canadian soils. Soil Sci., **46**, 307-321.

TOPPING, L. E. 1937 The predominant microorganisms in soils. I. Description and classification of the organisms. Zentr. Bakt. Parasitenk., II, **97**, 289-304.

THE EFFECT OF VARIOUS SUBSTANCES ON THE OXYGEN UPTAKE OF BLASTOMYCES DERMATIDITIS

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The lipid and carbohydrate content of *Blastomyces dermatiditis* has been described by Peck and Hauser (1938, 1940). Stewart and Meyer (1938) have studied the assimilation of glucose by this organism. There has been no report on its oxidative metabolism, and the purpose of this work was to study the factors affecting the oxygen uptake of this pathogenic fungus.

EXPERIMENTAL

The cultures were obtained from a clinical case of blastomycosis and were grown on Kelly's agar medium. They were used when 2–6 days old. The mat was scraped off the agar with a blunt glass rod, suspended in 100 ml. of sterile water and centrifuged for 5 minutes at 1500 r.p.m. The liquid was decanted and the solid ground in the tube with a thick glass rod to break up any masses. It was then resuspended in water and centrifuged again. This time the solid was taken up in 0.05 M phosphate buffer pH 6.7 so that 1.0 ml. of the packed organisms were suspended in 10.0 ml. of buffer. 0.5 ml. of this suspension was used in each Warburg vessel which contained a final volume of 2.0 ml. The fungus was used in the yeast form. The mycelial form, to which it tended to revert on a beef-extract glucose agar medium, has a lower oxygen uptake and oxidizes added substrates more slowly. The control uptake also decreases with the increasing age of the culture. A change of pH to 7.8 has little effect on any of the reactions described below.

Carbohydrates. Of the five sugars tested, glucose, mannose, fructose, arabinose, and rhamnose, only the first two are oxidized. They are oxidized at the same rate and take up 2 atoms of oxygen per molecule. Figure 1 shows the oxidation of glucose compared to that of pyruvate and lactate. Pyruvate takes up 1 atom of oxygen per molecule, lactate 2 atoms. The R.Q. of the control respiration averages 0.80, in the presence of glucose it rises to 0.96, and in the presence of pyruvate to 1.53. In order to determine whether one or more enzymes are responsible for the oxidation of these substances various drugs were added. The results are shown in figure 2 for glucose and pyruvate. It should be mentioned here that none of the sugars are fermented.

Although the organism used in this study is able to oxidize aliphatic aldehydes, aromatic aldehydes such as benzaldehyde and particularly anisaldehyde inhibit the control oxygen uptake. The latter causes a 15–25 per cent inhibition. It has no effect on the oxidation of glucose but definitely inhibits the oxidation of pyruvate and also of lactate. Mono-iodoacetate inhibits the control uptake 40 per cent, and almost completely inhibits the oxidation of glucose, but in the

presence of this drug pyruvate is apparently oxidized more rapidly. Sodium selenite acts in the same way. It is possible that in both cases pyruvate protects the organism in some way from the effect of the drugs, and that this is the cause of the apparent increase in the rate and amount of oxygen taken up. This protection is not due to a combination of the pyruvate with the drugs because it occurs to a similar extent when the relative concentrations of pyruvate and drug are widely varied. Both drugs inhibit the oxidation of lactate which, thus, like glucose, is unable to provide this protection. The selenite inhibition of the control respiration takes 1–2 hours to develop, depending on the concentration, and

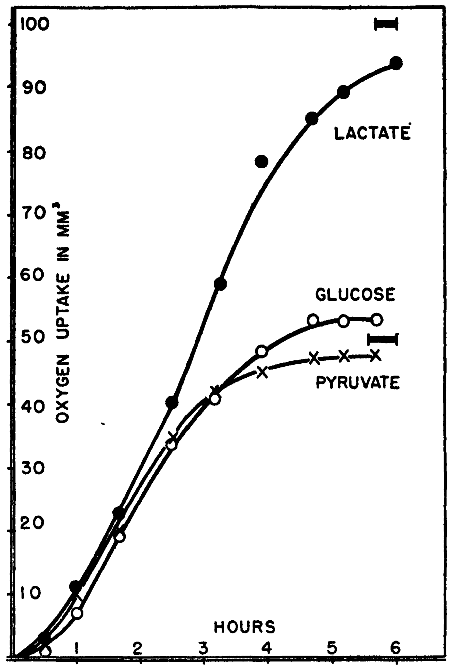

FIG. 1. OXIDATION OF 0.4 MG. EACH OF GLUCOSE, LACTATE AND PYRUVATE BY BLASTOMYCES DERMATIDITIS AT pH 6.7, 37°

The horizontal lines represent the theoretical uptake of 1 atom of oxygen per molecule of pyruvate and 2 atoms of oxygen per molecule of lactate and glucose. The control oxygen uptake has been subtracted.

finally may reach a value of 80 per cent. An acrid metallic odor develops after shaking selenite with the blastomycetes. This is not affected by the addition of any of the substrates tested, including methionine, but is completely absent if the selenite is incubated with the fungi anaerobically.

Fatty acids. The lower fatty acids are oxidized by these blastomycetes. In the oxidation of acetic acid 1 atom of oxygen is taken up and 1 molecule of CO_2 given off per molecule. The oxygen uptake tends to drift on further whereas the CO_2 production lags. The higher fatty acids behave very differently. Not only are they not oxidized, but they inhibit the control oxygen uptake, and also the

oxidation of added glucose, pyruvate, lactate, and acetate. Sodium oleate and palmitate are almost equally effective. Sodium stearate is less so, possibly because of its relative insolubility. It seems probable that the soaps cover the surface of the organisms and thus inhibit the penetration of oxygen and substrates. Figure 3 shows the effect of these soaps on the control respiration. Soy-bean lecithin in equivalent concentrations has no effect.

Amino acids. Immediately after washing the suspension of blastomycetes no free ammonia is detectable in it. After shaking in air for 5–6 hours in the War-

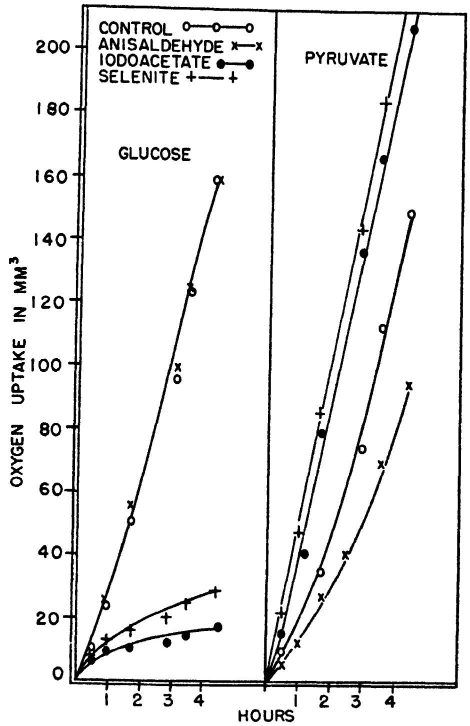

FIG. 2. EFFECT OF 1.0 MG. ANISALDEHYDE, 1.0 MG. MONOIODOACETATE, AND 0.2 MG. SODIUM SELENITE ON THE OXIDATION OF 2.0 MG. GLUCOSE AND 2.0 MG. PYRUVATE AT pH 6.7, 37°

The control oxygen uptakes have been subtracted. At the end of the experiment the control values were as follows: no drug, 380 mm³.; anisaldehyde, 328 mm³.; iodoacetate, 221 mm³.; and selenite, 111 mm³.

burg vessels appreciable amounts of ammonia accumulate. In order to determine whether the amino acids were the source of the ammonia, a number of them were added to the suspension. The results are shown in table 1. The addition of either the natural or the non-natural isomers markedly increases the oxygen uptake of the organisms. In many cases the uptake occurs more rapidly at the beginning in the presence of the non-natural isomer. For phenylalanine and methionine the uptake then proceeds at approximately the same rate in the presence of the two isomers, but for valine, alanine, and leucine the uptake in the

presence of the non-natural isomer eventually lags behind. The natural isomers of asparagin and aspartic acid not listed in the table have little or no effect.

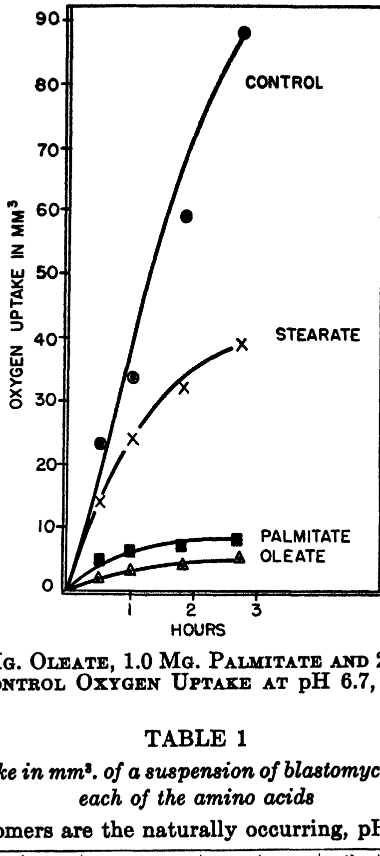

FIG. 3. EFFECT OF 1.0 MG. OLEATE, 1.0 MG. PALMITATE AND 2.0 MG. STEARATE ON THE CONTROL OXYGEN UPTAKE AT pH 6.7, 37°

TABLE 1

Increase in the oxygen uptake in mm³. of a suspension of blastomycetes in the presence of 1.0 mg. each of the amino acids

The l-isomers are the naturally occurring, pH 6.7, 37°

TIME	l-PHENYL ALANINE	d-PHENYL ALANINE	l-ALANINE	d-ALANINE	l-VALINE	d-VALINE	l-LEUCINE	d-LEUCINE	l-METHIONINE	d-METHIONINE	l-PROLINE	dl-PROLINE	l-TYROSINE	dl-SERINE	GLYCINE	PHENYL GLYCINE
hours																
0.30	3	12	0	5	0	0	2	3	3	7	3	0	3	12	2	0
1.00	8	25	0	10	0	0	7	9	8	15	8	0	6	18	3	0
1.30	19	38	0	12	0	0	16	18	18	29	46	17	14	29	4	0
2.0	37	57	0	17	9	3	33	32	38	49	93	56	29	50	6	11
3.0	85	108	12	32	57	19	54	46	63	69	142	95	49	72	36	28
4.0	168	186	46	54	112	40	77	60	88	88	209	150	76	105	129	40
5.0	294	294	93	74	201	60	104	73	119	106	287	207	109	134	163	53

Estimation of the free ammonia in the presence of the amino acids at the end of 5–6 hours showed that there was a small increase over that in the control. But although the oxygen uptakes caused by the amino acids were great enough to

account for their complete oxidative deamination, in no case was the theoretical amount of ammonia recovered. This suggested that the small increases found might be due to a stimulation of the control oxygen uptake, a "specific dynamic action" rather than to the utilization of the amino acids as substrates. This possibility was confirmed by the following facts. The increase of the oxygen uptake by the amino acids is not proportional to their concentration but is proportional to the number of organisms. This is shown in table 2 for phenylalanine. When a substrate such as glucose is added under the same conditions, the rate of oxygen uptake varies with the number of organisms but the same final end point is eventually reached. In the case of the amino acids not only are no definite end points reached, but the increase in oxygen uptake remains proportional to the number of organisms. Finally, at the end of the experiment, trichloracetic acid was added to the suspension, which was then filtered and aliquots taken for amino nitrogen determinations by the Van Slyke method. If no deamination of the amino acids occurred then the amino nitrogen found above that in the control should equal

TABLE 2

Increase in the oxygen uptake in mm³. of different amounts of blastomycetes in the presence of 0.4 mg. d-phenylalanine

TIME	ML. OF SUSPENSION		
	0.2	0.4	0.6
hours			
0.30	6	8	12
1.00	9	15	18
1.30	13	23	29
3.10	28	51	79
4.20	42	83	123
5.15	55	106	158
5.45	64	122	179

that added as amino acids. The results were as follows: l-phenylalanine, added 0.085 mg., found 0.086 mg.; d-phenylalanine, added 0.085 mg., found 0.075 mg.; d-alanine, added 0.158 mg. found 0.170 mg., glycine, added 0.187 mg., found 0.140 mg.

These facts indicate that the presence of the amino acids stimulates the oxidation of something in the cell and that they are not themselves oxidized when added under the conditions of the experiment. The source of the ammonia produced by the organisms is thus not determined. Stewart and Meyer have shown that the assimilation and production of ammonia by *Blastomyces dermatiditis* in different media can be affected by the presence of glucose. It is possible to show that the ammonia production by the control suspension under the conditions of the experiments described here is inhibited by glucose and certain other substances. After 5–6 hours in the Warburg vessels, trichloracetic acid was added, the suspensions filtered and aliquots taken, distilled from alkali and estimated colorimetrically with Nessler's solution. 0.072 mg. NH_3-N was produced by

the control; in the presence of 1.0 mg. of glucose 0.060 mg. was produced; 2.0 mg. glucose, 0.041 mg.; 2.0 mg. mannose, 0.034 mg.; 1.0 mg. acetate 0.064 mg.; 2.0 mg. acetate, 0.053 mg. Glucose and mannose very definitely suppress the ammonia production and acetate does also, but to a smaller extent. Pyruvate and lactate have little or no effect, but ethyl alcohol which is oxidized by the organisms has about the same effect as acetate. This suppression of ammonia production might be called a "protein sparing action."

The oxidation of succinic acid and the effect of cyanide. Succinic acid is slowly oxidized by the blastomycetes to fumaric acid. Malonate inhibits the oxidation completely. Fumaric acid does not act catalytically, nor does citric acid which is not oxidized. The oxidation of succinic acid indicates the presence of the cytochrome system. Cyanide completely inhibits the oxidation of all the added substrates, the effect of both the isomers of the amino acids and the ammonia production. Paradoxically, however, cyanide has a variable effect on the control oxygen uptake. The inhibition depends on the age of the culture used. M/100 NaCN inhibits the control uptake of a two-day-old and of a six-day-old culture, 40 and 5 per cent respectively. It is possible that cyanide does not readily penetrate into the cell. If this is so, it would be able to inhibit the oxidation of added substrates which may be oxidized on or near the cell surface but would have less effect on oxidation occurring within the cell. That the permeability of the cell becomes less with age is indicated by the fact that the latent period before the oxidation of added substrates takes place is longer the greater age of the culture used.

The effect of cyanide on the control respiration is apparently enhanced by the addition of oleic acid as indicated in the following experiment. A six-day-old culture was used. Just enough oleic acid was added to the suspension in one vessel to cause a 10 per cent inhibition of the control oxygen uptake. M/100 NaCN in another vessel caused a 5 per cent inhibition. But if both were added together a 55 per cent inhibition occurred. The explanation for this phenomenon is not clear but it is possible that cyanide penetrates more readily into the cell in the presence of oleate.

DISCUSSION

Although there have been numerous studies of the oxidative metabolism of non-pathogenic bacteria and fungi, similar studies on pathogenic organisms are relatively few. The present work is an attempt to outline one phase of the metabolism of a pathogenic fungus. The results show that it differs markedly in many respects from baker's and brewer's yeast. Until more data on pathogenic organisms accumulate it will not be possible to correlate these differences, but it is probable that such a correlation will eventually be made and that we may thereby attain a better understanding of the metabolic adaptations accompanying pathogenicity.

SUMMARY

1. Washed suspensions of *Blastomyces dermatiditis* oxidize added glucose, mannose, lactate and pyruvate.

2. The action of various drugs on the oxidation of these substances indicates that separate enzyme systems are involved.

3. Lower fatty acids are oxidized but higher fatty acids inhibit the control respiration and the oxidation of added substrates.

4. Free ammonia is produced by the suspensions. Both the natural and non-natural isomers of the amino acids increase the oxygen uptake but are not deaminated in the process.

5. Glucose and, to a less extent, acetate depress the ammonia production. Pyruvate and lactate are without effect.

6. Cyanide inhibits the oxidation of all added substrates including succinic acid but has comparatively little effect on the control respiration possibly because it fails to penetrate into the cell.

REFERENCES

PECK, R. L., AND HAUSER, C. R. 1938 Chemical studies of certain pathogenic fungi I. the lipids of *Blastomyces dermitiditis*. J. Am. Chem. Soc., **60**, 2599–2603.

PECK, R. L., AND HAUSER, C. R. 1940 Chemical studies of certain pathogenic fungi III. Further studies on the lipids of *Blastomyces dermatidis* and *Monilia albicans*. J. Biol. Chem., **134**, 403–412.

STEWART, R. A. AND MEYER, K. F. 1938 Studies in the metabolism of coccidioides immitis (Stiles). J. Infectious Diseases, **63**, 196–205.

BACTERIOLOGICAL STUDIES ON THE "NATURAL" FERMENTATION PROCESS OF PREPARING EGG WHITE FOR DRYING[1]

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Although the highest quality of commercial dried egg albumen has been produced for years, first in China and more recently in the United States, from fermented egg white, no adequate description of the fermentation has appeared in the bacteriological literature. Recently Stewart and Kline (1941) described rather completely the chemical changes taking place in egg albumen during experimental fermentation, but they apparently did not isolate or identify the bacteria to which they ascribed the changes reported.

From the manufacturers' viewpoint fermentation accomplishes at least two objectives. It removes enough of the carbohydrate so that the resulting dried product does not darken and lose quality during storage and it brings about a loss in viscosity or a thinning action facilitating handling and drying.

With the advent of the "Lend-Lease" program and the purchase by the Federal Surplus Commodities Corporation of huge quantities of dried egg products for shipment to England, the industrial significance of egg-white fermentation has been markedly increased.

Eight samples of commercially fermenting egg whites were obtained from various industrial concerns. When received, pH determinations and bacteriological examinations were made on each sample.

The bacteriological examinations consisted of making dilution plate counts using glucose agar, dilution smear plate counts using eosin-methylene-blue agar, smear plates of undiluted egg white on bismuth-sulfite agar and dilution plates in tomato-juice agar and glucose-tryptone agar.

The technique employed in making dilution smear-plates involved pouring the agar into the petri-dishes and allowing it to solidify and then placing 0.1 ml. of the diluted egg white on the surface of the agar near the center of the plate and distributing this uniformly over the surface of the entire plate with a sterile bent glass rod, using a rotary motion. All plates were incubated at 37.5°C. for 48 hours.

No aciduric types were recovered either from the tomato-juice or Bacto-tryptone agars. No growth was obtained from the smears of the undiluted egg white on the bismuth-sulfite agar, indicating absence of *Salmonella* and *Eberthella* types. The results of the dilution plate and the dilution smear plate counts are given in table 1.

[1] Agricultural Chemical Research Division Contribution No. 62.

The results in table 1 show counts well up in the hundreds of millions. Although the average count in the glucose agar dilution plates was somewhat higher than that found in the dilution smear plate counting procedure, using eosin-methylene-blue agar, the magnitude of the difference is not great enough to invalidate the conclusion that either *Aerobacter* or *Escherichia* types were present in such predominating numbers as largely to exclude other types. Where the colonies were of the *Escherichia* type the pH of the fermenting whites was found to be slightly below 6.0 whereas the pH of the whites from which the *Aerobacter* types were recovered was usually somewhat higher than 6.0.

Single colony isolations were made from the eosin-methylene-blue smear plates. The five *Aerobacter*-type isolations were subsequently found to be gram-negative rod forms capable of utilizing citrate. They fermented lactose and glycerol vigorously with the production of acid and gas. Acetyl-methyl-carbinol was produced. They were methyl-red-negative, indole-negative and did not liquefy gelatin. They were tentatively identified as strains of *Aerobacter aerogenes*. The *Escherichia*-type isolations were gram-negative rod forms capable of utilizing citrate and fermenting lactose with the production of acid and gas but failing to grow and produce gas in Eijkman's lactose broth at 45°C. in 48 hours. They were indole-positive and methyl-red-positive. They were tentatively identified as strains of *Escherichia freundii*.

TABLE 1

Results of bacteriological counts made on commercially fermenting egg white samples

SAMPLE NO.	pH AS RECEIVED (BECKMANN pH METRE)	DILUTION PLATE COUNTS ON GLUCOSE AGAR PER ML.	DILUTION SMEAR PLATES ON EOSIN-METHYLENE-BLUE AGAR	
			Count per ml.	Type of colony found
		millions	*millions*	
1	5.98	620	840	Escherichia
2	6.00	930	1,180	Aerobacter
3	5.90	270	710	Escherichia
4	5.95	410	260	Escherichia
5	6.15	840	1,210	Aerobacter
6	6.25	1,360	970	Aerobacter
7	6.20	2,110	830	Aerobacter
8	6.25	1,620	950	Aerobacter
Average........	6.08	1,020	856	

Using 3.6 ml. of a mixture of samples no. 1 and no. 2 as an inoculum, a batch of 1800 ml. of freshly broken-out strained egg white was fermented at 30°C. for 96 hours. Samples were taken periodically for pH determinations; formol titrations; sugar determinations, using Stiles, Peterson and Fred's (1926) modification of Schaffer and Hartmann's volumetric method; total protein nitrogen by precipitation with 2.5 per cent trichloracetic acid; and total bacterial count using the dilution plate method with glucose agar. The results of this study constitute a fairly complete confirmation of the data given recently by Stewart and Kline (1941). They are presented in figure 1.

From figure 1 it can be seen that there is a rapid increase in pH from 7.45 to 9.10 within the first 12 hours of incubation, independent of significant increases in bacterial numbers. It has been assumed that this increase is due to the breakdown of the $NaHCO_3$-$HHCO_3$ buffer system through the loss of CO_2. The initial increase in pH is immediately followed by a continuous and rather rapid decrease from 9.10 to 6.25 after 72 hours of incubation. This decrease in pH is accompanied by a decrease in the sugar content from an initial value of 23.2 mgm. per 10 ml. to 12.9 mgm. During this 72-hour period no change was observed in either

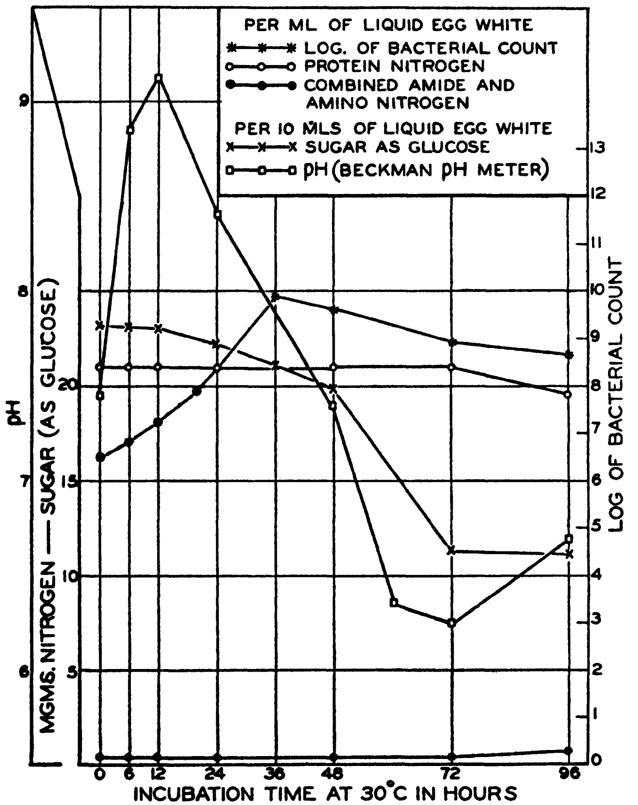

FIG. 1. BACTERIOLOGICAL AND CHEMICAL CHANGES OBSERVED DURING THE FERMENTATION OF EGG WHITE

the protein nitrogen or combined amino and amide nitrogen values. These remained constant at 21 and 0.4 mgm. per ml. respectively. Thus, within the first 72 hours it can be stated that there was no apparent breakdown of the egg white proteins. However, at 96 hours there was an increase in the combined amide and amino nitrogen value to 0.6 mgm. per ml. and a considerably larger decrease in the protein nitrogen value from 21.0 to 19.6 mgm. per ml. Between the 72- and 96-hour periods there was only an insignificant drop in the sugar content and only a slight decrease in the bacterial count. There was an abrupt increase in the pH value from 6.25 to 6.60. It should be pointed out that in the industry it is the

practice to stop the fermentation at approximately the 72-hour period and prepare the liquid white for drying. It would seem, therefore, that this fermentation is controlled to remove the carbohydrate fraction to a low level without digesting the protein.

The removal of sugar by the organisms does not appear to be complete. It may be that the method employed in measuring the sugar in this study measures some of the loosely bound sugar as well as the free sugar, and that actually all of the free sugar is utilized by the bacteria. This will be further investigated.

During this fermentation there is usually a separation into two liquid phases. The thin white phase accumulates in the bottom of the container and the thick white phase gradually rises as the pH decreases until all of the mucin with some of the mucoids accumulate on the surface in a gelatinous scum. This scum is removed from the liquid phase prior to drying. The increase in the volume of the thin phase at the expense of the thick phase as the pH decreases during a typical fermentation is shown in table 2.

TABLE 2

Increases in the volume of the thin white phase during a typical fermentation

INCUBATION TIME AT 30°C.	pH	VOLUME OF THIN WHITE PHASE	VOLUME OF THICK WHITE PHASE
hours		*ml.*	*ml.*
0	7.35	0	1800
6	8.85	205	1595
12	9.20	380	1420
24	8.75	450	1250
48	6.95	1390	410
72	6.20	1440	360*

* Foam.

Because it is apparent from preliminary water and alcohol solubility studies that the 360 ml. of foam as shown in table 2 contains most of the ova-mucin and a large share of the glyco-proteins, this physical separation must be considered as playing a definite role in the production of a commercial albumin of a high degree of purity. It appears that the gas produced by the bacteria tends to bring these protein fractions of the egg white to the surface, as the decrease in pH to their isoelectric points brings about a coagulation.

To obtain information on the regularity of this fermentation as a natural phenomenon, a series of laboratory fermentation studies was conducted.

In the first study in this series a batch of 9000 ml. of egg white from strictly fresh eggs was divided into five lots of 1800 ml. each and allowed to ferment at room temperatures (26° to 31°C.) in previously sterilized 2000 ml. cylinders. The initial bacterial count of this egg white was 230 per ml. which was rather low. The course of these five fermentations was followed by periodic pH measurements and dilution plate bacterial counts. As soon as the pH had dropped to 6.2, or lower, the fermentations were stopped and the white dried. The results of this study are given in table 3.

From these results it is apparent that the rate of the fermentation is much slower than was the case in the type fermentation shown in figure 1. Here, with an initial bacterial count of only 230 per ml., it required 288 hours with lot 1, 240 hours with lot 2, 192 hours with lot 3, 240 hours with lot 4, and 240 hours with lot 5 to bring about changes comparable to those obtained in 72 hours with the so-called type fermentation where the initial bacterial count was 2,180,000 per ml. A marked irregularity between the times required for the different lots to ferment was also apparent. Lot 1 required 96 hours longer than lot 3 and 48 hours longer than lots 2, 4 and 5.

Single-colony isolations were made from the final platings of each lot. One organism was found to predominate in each lot. In every case the predominating organism was a lactose fermenter capable of utilizing citrate. None of these

TABLE 3

"Natural" fermentation of low-count egg white from strictly fresh eggs

TIME	LOT 1		LOT 2		LOT 3		LOT 4		LOT 5	
	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.
hours										
0	7.25	2.3×10^2	7.24	2.3×10^2	7.25	2.3×10^2	7.25	2.3×10^2	7.25	2.3×10^2
6	8.80	2.3×10^2	8.90	4.5×10^1	8.85	1.2×10^1	8.95	9.0×10^1	8.90	2.6×10^2
24	8.85	4.2×10^2	8.95	2.4×10^2	8.90	2.8×10^2	8.80	4.0×10^2	8.90	3.0×10^2
48	8.82	2.3×10^3	8.80	2.8×10^2	8.80	1.2×10^3	8.90	3.1×10^2	8.90	3.1×10^3
72	8.85	3.2×10^2	8.80	3.4×10^2	8.80	8.0×10^4	8.90	4.6×10^2	8.90	4.4×10^3
96	8.90	4.2×10^3	8.90	4.2×10^3	7.90	1.0×10^6	8.95	2.7×10^5	8.90	1.6×10^4
120	8.90		8.90		7.00	5.4×10^7	8.95		8.90	
144	8.90		8.90		6.60	1.3×10^8	8.80		8.95	4.7×10^5
168	8.95	8.8×10^3	8.70	4.4×10^6	6.20	4.6×10^8	8.60	6.5×10^6	8.95	1.1×10^6
192	8.85	9.9×10^4	7.15	4.1×10^7	6.10	1.8×10^8	7.40	1.3×10^7	7.80	7.2×10^7
216	8.60	1.4×10^6	6.25	4.9×10^8			6.60	5.8×10^7	7.60	1.8×10^8
240	8.25	7.0×10^6	6.05	3.3×10^9			6.15	3.3×10^9	6.00	1.4×10^9
264	7.65									
288	6.05	5.4×10^8								

lactose fermenters liquified gelatin. The strains isolated from lots 1, 4 and 5 were tentatively identified as species of the *Aerobacter* genus and those from lots 2 and 3 as species of the genus *Escherichia* on the basis of the colony types produced on eosin-methylene-blue agar, and of tests made on cultures for the indole, methyl-red and Voges-Proskauer reactions.

In the second study of this series, the above experiment was duplicated in all details except for the introduction into the initial 9000-ml. batch of freshly separated egg white of a considerable quantity of egg shell contamination. This resulted in an initial bacterial count of approximately 150,000 per ml. The results of this study are given in table 4.

By comparing the results given in table 4 with those listed in table 3 it can be seen that a moderately heavy contamination of egg white by shell debris speeds up the rate of fermentation tremendously. With the five lots of white con-

taminated in this manner, the fermentation was apparently complete in 72 hours and the pH changes and bacterial count changes were practically identical for each time interval with all five lots.

Single-colony isolations were made from the dilution plates made at the 72-hour interval for all lots in this series. All isolations fermented lactose with the production of acid and gas. Streaks on eosin-methylene-blue agar showed colonies of the *Aerobacter* type only with lots 1, 2 and 4 and a mixture of *Aerobacter* and *Escherichia*-type colonies in lots 3 and 5. On the basis of findings from stains, cultures in citrate and the indole, methyl-red and Voges-Proskauer tests and cultures in gelatin and glycerol broth all *Aerobacter* types were tentatively classified as *Aerobacter aerogenes* and the *Escherichia* types as *Escherichia freundii*.

From the results obtained in a third study of this series of experiments it is apparent that a high initial total count does not necessarily assure rapid and

TABLE 4

"Natural" fermentation of high-count egg white from strictly fresh eggs contaminated with egg shells

TIME	LOT 1		LOT 2		LOT 3		LOT 4		LOT 5	
	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.	pH	Bacterial count per ml.
hours										
0	7.35	1.5×10^6	7.35	1.5×10^5	7.35	1.5×10^6	7.35	1.5×10^5	7.35	1.5×10^6
6	8.95		8.90		8.80		8.85		8.95	
18	8.72	1.2×10^7	8.85	7.3×10^5	8.85	1.1×10^6	8.85	5.8×10^6	8.60	1.1×10^7
24	8.40		8.50		8.75		8.75		8.20	
36	7.80	6.4×10^7	7.65	7.9×10^7	6.95	3.2×10^7	7.00	1.8×10^7	6.45	8.6×10^8
48	6.45		6.20		6.20		6.40		6.10	
72	6.05	1.2×10^9	6.15	1.6×10^9	6.20	2.6×10^9	6.25	4.7×10^8	6.10	3.7×10^9

uniform fermentation in the absence of a heavy initial seeding of either *Aerobacter* or *Escherichia* types. In this study two lots of strained egg whites of 1800 ml. each separated from storage eggs in such a manner as to avoid shell contamination but having counts after straining of 9,000,000 and 5,800,000 per ml. were allowed to ferment at room temperatures. These were sampled periodically for pH determinations and bacterial counts.

With these two lots pH changes took place slowly after the first six hours. After 182 hours the pH was still greater than 6.5 although the bacterial counts were very high even after 24 hours of the fermentation period. There was no foaming or physical separation into thin and thick phases as shown in table 2. Lot 1 developed a fishy odor and lot 2 developed an obnoxious putrid odor.

Single-colony isolations from the final dilution plates from these two lots failed to reveal the presence of either *Aerobacter* or *Escherichia* types. Three strains of gram-negative bacteria tentatively classified as belonging to the genus *Pseudomonas* were the only bacteria isolated from lot 1. From lot 2, two bac-

terial types were isolated; one was tentatively classified as belonging in the genus *Pseudomonas* and the other to the genus *Serratia*.

A fourth experiment in this series involved a study of the fermentation of thawed stored frozen whites in essentially the same manner employed in the preceding experiments. In this study a batch of 9000 ml. of stored frozen egg white was thawed for 24 hours at 20°C. It was then divided into five lots of 1800 ml. each and allowed to ferment in previously sterilized individual glass cylinders. Samples were removed periodically for pH measurements and bacterial counts.

By comparing the results obtained with those in table 4, it was found that the fermentation of the thawed and frozen whites proceeded at about the same rate and degree of regularity as that of fresh egg white that had been contaminated, or seeded, with egg shells. The initial pH of the frozen white was equivalent to that of the fresh white after six to twelve hours of fermentation. It can be assumed, therefore, that an amount of CO_2 is lost during the freezing and cold storage equivalent to that lost from freshly separated white in the first six to twelve hours of the fermentation process.

Single colony isolations were made from the final dilution plates of the five lots of fermented frozen whites in this study. Two isolations tentatively identified as belonging to the genus *Aerobacter* were recovered from lot 1. Three isolations, one of which was tentatively identified as belonging to the genus *Aerobacter* and two to the genus *Pseudomonas*, were recovered from lot 2. One isolation from lot 3 was classified as belonging to the genus *Aerobacter* and another to the genus *Pseudomonas*. Lot 4 yielded a strain of *Escherichia freundii* and a strain of *Proteus*, and lot 5 one strain of *Aerobacter* and one of *Alkaligenes*.

The completely fermented thawed frozen whites yielded, therefore, a considerably greater variety of bacterial types than was found in any of the fermented fresh whites when strains of either *Aerobacter* or *Escherichia* types predominated. This may be due to a decrease in the viability of the *Aerobacter* and *Escherichia* types during storage at freezing temperatures to the extent that they are no longer able completely to overgrow the other types present. Further work will be necessary to establish this presumption.

A fifth study of this series was made in a similar manner with egg whites freshly separated into sterile cylinders, with as little contamination as possible from the egg shells or hands of the laboratory worker, from shell eggs received from six different locations in the United States. These were allowed to ferment at room temperatures and sampled periodically, as in the previous studies, for pH measurements and bacterial counts.

The results obtained were essentially a duplication of those given in table 3 for low-count egg white except that the irregularity between the times required for the different lots to ferment was more pronounced. This result is precisely what might have been expected since the initial count was very low in all but 2 of the lots. With one lot fermentation in the true sense of the word did not occur within 398 hours. Single colony isolations showed that in this case there was a combination of a very low initial count and the absence of *Aerobacter* and

Escherichia types. Only one organism was isolated and this was tentatively identified as belonging to the genus *Pseudomonas*.

Isolations from the final dilution plates of 2 lots that fermented fairly rapidly and normally revealed the predominance of *Escherichia* types in one case and a mixture of *Escherichia* types with a strain of *Proteus* in the other. Isolations from the other 3 lots in which the fermentations were more or less delayed yielded a strain of *Serratia* and one of *Aerobacter* in one instance, two strains of *Proteus* and one of *Aerobacter* in another and two strains of *Pseudomonas* with one of *Proteus* in the other.

All lots of egg whites fermented in these studies were subsequently dried in a "Hurricane" drier at 110°F. for 72 hours along with lots of unfermented whites as controls. With this type of drying a granular dried white resulted. With those lots in which the *Aerobacter* and *Escherichia* types predominated, a bright crystalline clear light-colored product was obtained as compared to a dull amorphous-type product where organisms of the *Proteus*, *Serratia*, and *Pseudomonas* genera were present in significant numbers. The fermented dried whites in all cases retained their original color in storage experiments as compared to the unfermented dried white control samples. The latter materially darkened after four months' storage in the laboratory at room temperature.

Because fermentations from which proteolytic organisms were isolated produced amorphous dingy granular albumin on drying, it may be that the accurate stopping of the fermentation at the critical point where the pH reaches the minimum value of 6.25 and the sugar value reaches a relatively constant low level prior to the appearance of proteolytic changes is necessary in order to control the texture and color of the resulting dried product.

SUMMARY

1. Bacteriological studies on eight samples of commercially fermenting egg white revealed the presence of bacteria of the genera *Aerobacter* or *Escherichia* in such predominating numbers as practically to exclude other bacterial types.

2. Chemical studies on the fermentation of egg white separated from fresh eggs and inoculated from commercially fermenting egg white showed that during the first 72 hours of fermentation there was a production of acid at the expense of the sugar. During this time no appreciable hydrolysis of protein occurred. Marked evidence of proteolysis was found at 96 hours. By this time the sugar level had been reduced to a relatively low constant value. Protein hydrolysis was accompanied by a pronounced increase in the pH value.

3. Egg white separated from fresh eggs with a low initial bacterial count fermented slowly and irregularly.

4. Egg white separated from fresh eggs with a relatively high initial bacterial count, due to shell contamination, fermented rapidly and regularly.

5. Egg white separated from storage eggs with a high initial bacterial count, essentially independent of shell contamination, but originating from the interior of the egg, fermented slowly and incompletely. No bacteria of either the *Aerobacter* or *Escherichia* genera were found in these egg whites.

6. Thawed frozen egg white fermented rapidly and regularly. The course of the fermentation did not appear to be essentially different from that observed with egg white from fresh eggs contaminated with egg shells.

7. Dried fermented egg white retained its original color during storage in the laboratory at room temperatures for four months whereas dried unfermented egg white turned dark reddish brown.

8. Batches of fermented egg white in which *Aerobacter* and *Escherichia* types predominated during fermentation were found to yield a bright, crystalline, granular product on drying. Batches of fermented egg white in which such proteolytic bacteria as strains of the genera *Proteus*, *Serratia*, and *Pseudomonas* persisted throughout the fermentation period, yielded a dull, dingy, and amorphous product upon drying.

REFERENCES

Stewart, G. F., and Kline, R. W. 1941 Dried egg albumen. I. Solubility and color denaturation. Proc. Inst. of Food Technologists, pp. 48–56. Pittsburgh, Pa.

Stiles, H. R., Peterson, W. H., and Fred, E. B. 1926 A rapid method for the determination of sugar in bacterial cultures. J. Bact., **12**, 427–434.

A TEST FOR SEXUAL FUSION IN BACTERIA[1]

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The question—do bacterial species have a sexual stage with consequent reduction, segregation and recombination of their inheritance?—is fundamental to an understanding of pathogenicity, of virulence changes, or any other genetic variation. If bacteria have sexual stages, rapid segregation and recombination of any heterozygous inheritance into new variation would be possible. If reproduction is strictly asexual then inherited variation is limited to the less frequent process of mutation or possibly somatic segregation. A critical technique to throw light on this question is urgently needed.

Character differences within a bacterial species furnish the necessary material for such a test. These characteristics may be yellow vs. white colony color, rough vs. smooth colony type, cataphoric movement to + or − poles, or any other character pairs for which it is possible to establish strains giving consistently the same progeny types,

Two types, i.e., yellow and white colony color, are carried in separate pedigree cultures in sufficient serial platings to demonstrate that these types will give nothing but yellow colonies on the one hand or white colonies on the other. The two types are seeded into the same sterile broth tube or inoculated into a suitable host to give opportunity for sexual fusion to occur between bacteria of the two types. At successive time intervals bacteria, which may be the resultant of the fusion of the two types, are plated out. Such bacteria, i.e., those resulting from fusion, should be heterozygous for the differential characteristics and should segregate for these types in succeeding generations. A second plating is made to test this point. If sexual reproduction by fusion of two different bacteria were reasonably frequent, individual colonies should be obtained on the first plating which could be demonstrated to segregate in the second plating for *both* yellow and white progeny. Observing these types would presumably only necessitate examination of sufficient numbers of bacteria from the mixed culture. If reproduction is only by simple fission, however, only yellow progeny should be obtained from yellow colonies selected in this generation and only white colonies from white. A differential is consequently offered for the two methods of reproduction especially if the experiments are repeated on several different character pairs. This technique is not greatly different from that of Sherman and Wing (1937) save for the emphasis which we place on establishing the purity of the initial strains and a progeny test.

Because of its own interest and significance this technique has been applied to

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strains of *Phytomonas stewartii* (E.F.S.) Bergey *et al.* which differed in color, yellow vs. white; in colony type smooth vs. rough; and in pathogenicity.

Bacterial stocks of white strains and yellow strains were tested for purity by 5 successive platings from single colonies. Cultures were taken from these known pure white and pure yellow strains and inoculated into the same nutrient broth. These broth cultures of mixed bacteria were grown at summer room temperature. The data are shown in table 1. Samples for plating were taken at 1, 3, 7, 14, 21, 28 and 60 days. A total of 1927 yellow colonies and 145 white colonies from mixed broth cultures were plated individually onto agar with a smear technique. The mixture of A87 × 105 (see table 1) was further differentiated by being marked by the characters smooth and rough. The 363 yellow colonies of this derivation were smooth, the 35 white colonies were rough. The colonies on each plate were observed at 16 diameters for any segregation of yellow vs. white,

TABLE 1

Progeny of bacterial strains where opportunity for sexual fusion is present

DURATION OF TIME WHEN FUSION COULD TAKE PLACE	PARENT STRAINS MIXED TOGETHER—STRAIN NO. AND PHENOTYPE								
	S15 and 105			A87 and 105			101 and 105		
	Rough yellow	Rough white		Smooth yellow	Rough white		Rough yellow	Rough white	
	Types recovered from progeny								
	Pure yellow	Pure white	Mixed	Pure yellow	Pure white	Mixed	Pure yellow	Pure white	Mixed
days									
1	67	5	2						
3	125	5		54	5		50	5	
7	70	5		40	5		140	10	
14	40	10		137	10		100	5	2
21	263	5		42	5		186	15	
28	80	5		30	5		100	5	
60	80	20		60	5		263	15	
Total....	725	55	2	363	35		839	55	2

of smooth vs. rough, or both characters as the case might be. With 4 exceptions all colonies were pure for type. The exceptions were all yellow colonies that showed a segregation for white colonies in the second plating. From 75 to 125 colonies from each of the segregating plates were further plated. No colony from the 4 segregating plates showed further segregation. It is known that bacteria of this species sometimes stick together, the rate being *ca* 1 per cent as determined by sectioning technique, McNew (1938). The appearance of 4 mixed colonies in 2072 colony isolations is consequently not unexpected. These results incidentally confirm the infrequency with which a single colony is produced from two distinct strains of this species.

The data of table 1 are concordant with the view that under these experimental conditions *P. stewartii* does not have a sexual fusion similar to that of higher forms. Sexual fusion is still possible, however, if we postulate chromatin reduc-

BACTERIOSTATIC AND BACTERIOLYTIC PROPERTIES OF ACTINOMYCETES[1]

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Investigations on the antimicrobial properties of actinomycetes can be divided into two groups: (a) those dealing with the bacteriostatic and bactericidal activities of these organisms, and (b) those concerned primarily with their bacteriolytic properties. In both cases, the possible practical applications have been kept in mind. The hope of isolating specific antibacterial substances potentially useful for the control of infectious diseases of man, animals, or plants has been general. Interest in the lytic agents is dominated by the possible use of bacterial lysates for vaccination purposes.

HISTORICAL

Since the literature concerning the antibacterial action of actinomycetes (and other microorganisms) has recently been fully reviewed by Waksman (1941), it will be sufficient to recall here that antibiotic properties are widely distributed among these organisms, as shown by the work of Nakhimovskaia (1937), Krassilnikov and Koreniako (1939), Alexopoulos (1941), Waksman, Woodruff and Horning (1941) and Welsch (1942). At the present time, two powerful antimicrobial agents have been obtained from cultures of actinomycetes. The first was designated as actinomycin; it was obtained from *Actinomyces antibioticus* (Waksman and Woodruff, 1940a), and has been purified and crystallized (Waksman and Woodruff, 1940b, Waksman and Tischler, 1942). The second, designated as streptothricin, was obtained from a strain of *Actinomyces lavendulae* (Waksman and Woodruff, 1942). The bacteriolytic properties of actinomycetes, with which the present paper is mostly concerned, are less well understood than their bacteriostatic activities.

The credit for having first recognized the ability of actinomycetes to destroy microbial cells is generally given to Gasperini (1890), who observed, in the course of his classical researches on *Streptothrix foersteri* Cohn, that the filaments of this organism may destroy the cell-membrane of several bacteria and fungi. The first experimental study of the bacteriolytic action of actinomycetes, however, did not begin until 30 years later. Lieske (1921) not only described the antagonistic effects of actinomycetes on *Staphylococcus aureus*, but also reported the dissolution of various dead or living bacteria, incorporated in water-agar, by actinomycetes streaked on the surface of this medium.

In the course of investigations on bacteriophage, Gratia and Rhodes (1924)

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described the phenomenon of isophagy of staphylococci. This observation led Gratia and Dath (1924–1931) to the discovery, quite independently of Lieske, of the bacteriolytic action of actinomycetes; it was found that these organisms are able to dissolve many heat-killed or living pathogenic bacteria suspended in water. The lysates, although much less toxic than the non-treated suspensions, were very good antigens. Accordingly, Gratia (1930–1934) employed these "mycolysates" as a therapeutic agent in several infectious diseases of man. Rosenthal (1925) also observed the lysis of *Corynebacterium diphtheriae* by an actinomyces contaminating the Loeffler's medium plate.

These cases of bacteriolytic action of actinomycetes are closely related to the type of activity exhibited by a species of *Actinomyces* belonging to the *A. albus* group, which has been extensively studied by the writer (Welsch, 1936–1939, 1937, 1938, 1941)[3].

A summary of the antibacterial properties of this organism follows.

Actinomyces G grows readily in liquid or agar mineral media containing heat-killed or chemically killed gram-negative or gram-positive bacteria, or living gram-positive bacteria. The bacterial suspensions become clarified after a few hours in the case of heat-killed gram-negative bacteria and only after 2 to 3 days in the case of either heat-killed or living gram-positive organisms. It is essential that the bacterial suspensions be inoculated with a sporulating broth-culture of *Actinomyces* G and that a small amount of the culture medium be transferred together with the organism. The antagonist grows very scantily in suspensions of living gram-negative bacteria where it produces no lysis at all. It dissolves killed bacteria suspended in nutrient-broth but does not affect living bacteria to any extent under those conditions (Welsch, 1936–1939).

Sterile filtrates of broth-cultures of *Actinomyces* G, obtained after sporulation, were designated as "actinomycetin" (Welsch, 1937); they dissolved in a few hours suspensions of heat-killed gram-negative bacteria and in 24 hours those of heat-killed gram-positive bacteria. They had no action, however, on most living gram-positive bacteria, although partial lysis of *Klebsiella pneumoniae*, *Streptococcus hemolyticus* and *Staphylococcus aureus* was obtained. They had no action on any living gram-negative organism so far tested.

The properties of the lytic agent are those of a protein (Welsch, 1936–1939) and of an enzyme (Welsch, 1938). Concentrated preparations of the lytic agent have been obtained (Welsch, 1941), but, though active upon killed bacteria, they were inactive when tested on most living organisms.

By the use of the cross-streak method, it has been shown that *Actinomyces* G has a bacteriostatic effect against many gram-positive bacteria but not against any of the gram-negative organisms examined (Welsch, 1942).

Crude actinomycetin (Welsch, 1937) has no bacteriostatic activity, but con-

[3] The identity of this organism with any one member of the highly heterogeneous *A. albus* group (Duché, 1934) has not yet been established. The organism will, therefore, be designated in the present paper as *Actinomyces* G, in homage to Dr. A. Gratia who was one of the first to undertake a systematic study of the occurrence of bacteriolytic organisms in nature.

centrated preparations, added to nutrient-agar, prevented the growth of several gram-positive bacteria (Welsch, 1941). Ether-extracts of actinomycetin had a notable bactericidal action on gram-positive bacteria suspended in an inorganic medium; this action was greatly reduced in the ordinary complex culture-media. The active agent appeared to be a lipoid, probably a fatty acid. Part of it originated from the culture-medium, since similarly active extracts were obtained from sterile media; another part of it was, undoubtedly, produced as a result of the growth of the *Actinomyces* (Welsch, 1941). "Actinomycin B" produced by *Actinomyces antibioticus* is, presumably, of a similar nature (Waksman and Woodruff, 1940b).

Bacteriolysis of living bacteria by *Actinomyces* G was visualized as a two-step reaction: first, the susceptible cells are killed by the selectively bactericidal lipoid; second, those dead cells are then dissolved by the bacteriolytic enzyme (which alone is responsible for the lysis of heat-killed bacteria). The phenomenon does not take place in complex culture-media, since the bactericidal action of the lipoid is greatly impaired under those conditions, and the presence of living *Actinomyces* is generally necessary, since free lipoid should be secreted in the susceptible suspension (Welsch, 1941).

Another type of bacteriolysis by actinomycetes, especially by *Actinomyces violaceus*, was reported recently by Krassilnikov and Koreniako (1939) and by Kriss (1940). The active principle involved differs notably from the lytic agent of actinomycetin, since it is relatively thermostable, resistant to ultra-violet radiations, easily adsorbed on various substrates and most abundantly produced in synthetic media. Like the lytic agent of actinomycetin, it is soluble in water, insoluble in organic solvents, and less active in acid media. This agent was said to resemble egg-white lysozyme (Fleming, 1922); however, the justification of this assumption may be questioned. The nature of the active principle remains wholly unknown, whereas lysozyme has been purified and crystallized (Meyer *et al.*, 1936; Roberts, 1937; Abraham, 1939). The bacterial constituent acted upon by the new agent is not known, whereas the component susceptible to lysozyme has been isolated (Epstein and Chain, 1940) and shown to be a polysaccharide. Staphylococci are the organisms most susceptible to the new agent which has no selective action on *Micrococcus lysodeikticus*; the latter is the best test-organism for lysozyme, which has virtually no action on staphylococci (Thompson and Khorazo, 1935). Lysozyme is further resistant to acidification, even at a high temperature (Thompson, 1941).

CULTURES AND METHODS

Source of cultures studied. Altogether, 275 strains of actinomycetes were used. These may be divided into two groups: first, 111 cultures taken at random from a series of 250 strains freshly isolated from various soils by Mrs. E. S. Horning, in this laboratory; second, 164 strains, including representatives of the genera *Actinomyces*, *Proactinomyces* and *Micromonospora*, isolated from various soils, marine material, potatoes, lake-mud, etc., were obtained from the type culture

collection of the N. J. Agr. Exp. Station. The latter strains had been kept in the collection for periods of time ranging from a few months to more than 25 years.

Cultivation of the actinomycetes. Stock-cultures were maintained on slants of beef-extract-peptone-agar and of modified Czapek's-agar. Spore-suspensions in sterile tap-water were obtained from such slants for the inoculation of all experimental cultures. The actinomycetes to be tested for bacteriolytic activity were grown at 30°C. in 25 ml. portions of broth. Cultures on nutrient-agar plates or on starch-tryptone-agar[4] in Blake bottles were used in the tests for bacteriostatic activity.

Test-organisms. A strain of *Escherichia coli*, isolated by the writer from the urine in a case of cystitis, was used as a typical representative of gram-negative organisms. A strain of *Staphylococcus aureus*, isolated from a carbuncle, was used as a typical representative gram-positive organism. A collection strain of *Bacillus subtilis* and one of *Sarcina lutea* were widely used in the tests of bacteriostatic activity. A strain of *Micrococcus lysodeikticus*, originally obtained from Dr. Fleming, was used for lysozyme tests. In addition, a number of bacterial strains of various origin were used and will be listed in the description of the corresponding experiments.

The stock-cultures of the test organisms were maintained on beef-extract-peptone-agar slants or on serum-agar (pneumococci, streptococci, *Neisseria*, corynebacteria). Suspensions of the organisms in sterile tap-water were made from 24- or 48-hour-old slants for the inoculation of all experimental cultures.

The bacteria to be tested were grown for 24 or 48 hours at 30° or 37° on 25 ml. beef-extract-peptone-agar or serum-agar in Blake bottles. The cells were then aseptically suspended in 5 ml. of sterile mineral salt solution (Czapek without sugar). In experiments with living test-organisms, this heavy bacterial suspension was used directly after suitable dilution. In experiments with heat-killed test-organisms, the bacterial suspension was first inactivated either at 65° for 30 minutes or in a boiling water-bath for five minutes, then suitably diluted.

Tests for lysis by growing actinomycetes. Three slightly different methods were used, and records made after 18, 24, 48, and 72 hours incubation, at 30°C.

(a) The heavy suspension of test-organisms, either fresh or heated, was diluted with sterile mineral solution until a turbidity equivalent to that of 10^8 cells per ml. was obtained. The Pulfrich-Zeiss nephelometer was used for standardization (Welsch, 1938). The dilute bacterial suspension was aseptically distributed (4.5 ml. portions) in test-tubes and each tube inoculated with 0.5 ml. of a broth-culture of the actinomyces. Evidence of lysis was sought by direct examination or by nephelometric measurements. A slightly different procedure was used in the tests with living organisms. The mineral solution was distributed in 250 ml. Erlenmeyer flasks (25 ml. portions), sterilized, inoculated with 0.5 ml. of a broth-culture of the actinomyces, and incubated at 30° for 48

[4] Bacto-tryptone, 10 gm.; corn starch, 5 gm.; NaCl, 0.2 gm.; K_2HPO_4, 0.2 gm.; bacto-agar, 10 gm.; Tap-water, 1000 ml.

hours. Thereafter, a suitable amount of the heavy bacterial suspension was aseptically added to each flask to obtain the desired turbidity. After further incubation, the extent of lysis was determined.

(b) The heavy bacterial suspension was suitably diluted in melted and cooled soft (0.5%) synthetic agar. This was next distributed in test-tubes, in 5 ml. portions, and allowed to solidify. Each tube was inoculated with 0.2 ml. of a broth-culture of the actinomycetes and incubated. Development of the actinomycetes on the surface, the presence of a clarification zone underneath, its depth and intensity, and the presence of alternately clear and dark rings were recorded.

(c) The heavy suspension was suitably diluted in melted and cooled synthetic agar (1.5%); this agar was then poured into Petri dishes, in 15–20 ml. portions, and allowed to solidify. Each plate was inoculated with four different actinomycetes, streaks about 2 cm. in length being made. After incubation at 30°, growth of the actinomycetes, formation and width of clear zones around the colonies were determined.

Tests for lysis by soluble products of actinomycetes. For this purpose, either liquid or solid medium was used. The heavy suspension of fresh or heat-killed bacteria was suitably diluted in M/15 phosphate buffer of pH 8.0; the dilute suspension was then aseptically distributed in test-tubes (4.5 ml. portions) and warmed to 38° in a water-bath. To each of several tubes was then added 0.5 ml. of a paper- or Seitz-filtrate of a broth-culture of the actinomycetes previously warmed to 38°C.; at this moment, the time was recorded with a stop-watch. The mixture was kept at 38° in a water-bath; samples were removed from time to time, rapidly cooled in ice, and examined in the nephelometer. With heat-killed gram-negative organisms, samples were examined during a period of 20 to 120 minutes and the mathematical relations described by the writer were used to express the activity in "mycolytic units" (Welsch, 1938). With heat-killed gram-positive bacteria and with living microorganisms, the examination of the samples was extended through a period of 24 to 48 hours. In each case, bacterial suspensions without filtrate and bacterial suspensions with boiled filtrate added were used as controls. When a solid medium was used, bacterial-agar plates were prepared as described above. Fragments of sterile agar were taken in the vicinity of the actinomycetes grown on various solid media and transferred to the bacterial-agar medium. After incubation at 37°, presence and width of the clarification zone surrounding the bits of agar were recorded.

Test for bacteriostatic action of growing actinomycetes. The actinomycetes to be tested were streaked across a plate of nutrient-agar and incubated for 48 hours at 30°C. A loopful of a suspension of each test-organism in tap-water was streaked at a right angle from the periphery of the plate toward the culture of the actinomyces. Three test-organisms were generally used on every single plate. After further incubation for 24 hours at 30° or 37°, the presence and the width of a sterile zone in the proximity of the *Actinomyces* were recorded.

Test for bacteriostatic action of soluble products from actinomycetes. Paper-filtrates from broth-cultures as well as extracts from tryptone-starch agar-

cultures were tested. The extracts were obtained by shaking the cultures vigorously in 25 ml. of sterile tap-water, allowing to stand for two or more hours and filtering aseptically through paper. To test the activity, varying quantities of the filtrates were diluted in 10 ml. nutrient-agar, which was poured into a Petri dish and allowed to solidify. The test-organisms, four per plate, were streaked on the treated medium, tap-water suspensions of the bacteria from 24-hour-old slants being used. Occurrence of normal or reduced growth or absence of growth was recorded by comparison with the type of growth obtained on the control medium.

EXPERIMENTAL RESULTS

Type of bacteriolytic activity produced by actinomycetes

From a series of 250 cultures of actinomycetes, freshly isolated from various soils, 111 strains were taken at random, inoculated on living-staphylococcus mineral-agar and incubated for 6 days. At the end of this period, 39 strains had produced a clarification of the surrounding medium, whereas 72 cultures were inactive. For more detailed study, 42 cultures were selected from the above 111; they included 21 of the most active, six of the least active and 15 of the inactive strains. *Actinomyces* G was added to the series for the purpose of comparison; in all tests, this organism was as active as, or more active than, any of the strains studied.

The actinomycetes were inoculated in nutrient-broth and examined for bacteriolytic action on heat-killed *E. coli* and *S. aureus*, as well as on living *E. coli* and *S. aureus*. With each of these four substrates, the three methods above described were employed. On the basis of the results obtained, the strains were arbitrarily classified as highly active, moderately active or inactive. A final classification of the strains, on the basis of their action on each substrate, was next made by a careful comparison of the results recorded in the three different tests. Thus, organisms falling in the same activity-group in all three tests or in two of the three tests were finally classified in that group. Organisms falling in a different group in each test were classified in the middle group, i.e. as moderately active. At last, a statistical comparison of the ability of actinomycetes to attack the different bacterial substrates was made by the four-fold table and "chi-square" method (Fisher, 1930; Pearl, 1930).

The tests in soft mineral-agar were made with transfers from two-day-old cultures of the actinomycetes; when killed bacteria were used, they were inactivated in a boiling water-bath for five minutes. Tests in mineral agar were made with transfers from eight-day-old broth-cultures of the actinomycetes and tests in mineral-solution were made with transfers of eleven-day-old cultures; when killed bacteria were used in these two tests, they were inactivated at 65°C. for 30 minutes.

Before testing for lysis of living bacteria, it was thought desirable to demonstrate that the test-organisms survived long enough in the basic mineral solution to permit experiments extending over a period of three days. For this purpose, suspensions of *S. aureus* and *E. coli* in mineral-solution were incubated at 30°

for four days and colony-counts by the plate method made daily. The number of viable cells in suspension was calculated from the average number of colonies counted on five replicate plates. It was found that the test-organisms survived well within the limits of time needed for the experiments.

Growth of all actinomycetes tested was obtained on heat-killed *S. aureus* and *E. coli* as well as on living *S. aureus*; on the contrary, only a few strains grew, and very scantily, on living *E. coli*.

With heat-killed suspensions in soft-mineral-agar, evidence of lysis was observed within 18 hours after inoculation with some of the actinomycetes; with other strains, however, lysis was not observed until after incubation for two days. The zone of lysis was always very sharp and, after three days, its depth varied from six to 20 mm. In many cases, there was a second sharp zone of partial lysis, five to 25 mm. deep, under the clear zone. With living *S. aureus* in soft-mineral-agar, evidence of lysis after 18 hours was observed only with a few strains but was commonly found after 48 hours; the depth of the clear zone, after three days, varied from five to 20 mm. The presence of a zone of partial lysis underneath the region of complete clarification was seldom observed, but the production of clear zones alternating with darker rings was of common occurrence. The phenomenon was highly dynamic, since the position of the deeper dark bands changed progressively, whereas the dark rings closest to the surface gradually faded away and eventually disappeared. This phenomenon, which is probably related to the, as yet unexplained, Liesegang's rings, was never observed with heat-killed bacteria. This suggests that it is the result of some biological activity of the test-organism.

In mineral-agar, evidence of lysis was generally not observed before 48 hours. The clear zone had a width of two to 12 mm. with heat-killed bacteria, and of two to 10 mm. with living *S. aureus*. In this last case, the production of dark rings was occasionally noted, but this phenomenon was less frequent and less conspicuous than in soft-agar.

In mineral-solution, a decrease of turbidity of the heat-killed suspensions was observed after a few hours' incubation, in a small number of cases. With most of the active organisms, however, evidence of lysis was obtained only after 24 to 48 hours; after that time, the effect produced varied from a partial clarification to a complete clearing. In the case of living *S. aureus*, the actinomycetes were incubated in mineral-solution for 48 hours before the introduction of susceptible cells; evidence of lysis was generally found after 18 or 24 hours; the lysis remained partial or, most often, became complete after 48 hours.

Not a single strain of the many actinomycetes tested was found able to produce lysis of living *E. coli* by any of the methods used.

The distribution of the different types of bacteriolytic properties of actinomycetes is indicated in table 1. This table shows that about the same number of strains fell into each of the three activity-groups, whether the test-organism used was heat-killed *E. coli*, heat-killed *S. aureus* or living *S. aureus*. The results do not show, however, whether the organisms placed in corresponding groups for their action on different substrates are the same or not. The fourfold

tables demonstrate, respectively, the relation between activity of actinomycetes on heat-killed *E. coli* and on heat-killed *S. aureus* (table 2), and between their action on heat-killed *S. aureus* and on living *S. aureus* (table 3). It may be

TABLE 1

Lytic action of actinomycetes on various bacterial substrates

SUBSTRATE	ACTIVITY	NUMBER OF STRAINS ACCORDING TO ACTIVITY AND METHOD			
		Soft-agar	Agar	Solution	Final classification
Heat-killed *E. coli**	++	12	16	19	12
	+	20	12	7	17
	0	10	14	16	13
Heat-killed *S. aureus*†	++	15	11	8	12
	+	17	18	10	17
	0	10	13	24	13
Living *S. aureus*‡	++	7	7	24	8
	+	23	14	3	20
	0	12	21	15	14
Living *E. coli*	++	0	0	0	0
	+	0	0	0	0
	0	42	42	42	42

* In soft agar: ++ = clear zone 3–10 mm. deep in 18 hours; 14–20 mm. in 3 days; + = zone appearing after 48 hours; 5–12 mm. deep in 3 days; 0 = no clarification in 3 days.

In agar: ++ = clear zone 3 mm. in 2 days; 7–12 mm. in 3 days; + = Zone smaller than 6 mm. wide in 3 days; 0 = no clarification in 3 days.

In solution: ++ = complete clarification in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.

† In soft agar: ++ = lysis noticeable after 18 hours; zone 10–20 mm. deep in 3 days; + = lysis noticeable after 48 hours; zone 4–10 mm. deep in 3 days; 0 = no lysis in 3 days.

In agar: ++ = zone 4–10 mm. wide in 3 days; + = zone smaller than 4 mm. in 3 days; 0 = no lysis in 3 days.

In solution: ++ = complete clearing in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.

‡ In soft agar: ++ = lysis noticeable in 18 hours; zone 10–20 mm. in 3 days; + = lysis noticeable in 48 hours; zone 4–10 mm. in 3 days; 0 = no lysis in 3 days.

In agar: ++ = zone 3–10 mm. in 3 days; + = zone 1–3 mm. in 3 days; 0 = no lysis in 3 days.

In solution: ++ = complete lysis in 2 days or less; + = partial lysis in 2 days; 0 = no lysis in 3 days.

seen from these tables that most organisms capable of lysing one substrate are also able to dissolve the others. The statistical significance of the results may be ascertained by the "chi-square" method, which shows, in effect, that the experimental data are clearly opposed to the hypothesis of independence of the properties tabulated.

In the absence of a pure preparation of the lytic agent, it is, of course, impossible to know, by direct experiment, whether a single lytic principle is responsible for the action observed on the three different bacterial substrates used. The statistical evidence brought forward indicates that there is no reason, in the absence of contrary experimental data, to assume that different specific lytic agents are responsible for the action of any strain on different bacterial substrates.

TABLE 2

Fourfold table showing the relation between bacteriolytic action on heat-killed E. coli and on heat-killed S. aureus

LYSIS OF E. COLI	LYSIS OF S. AUREUS		TOTAL
	+	0	
+*	26	3	29
0	3	10	13
Total	29	13	42

Chi square $= \frac{[(26.10) - (3.3)]^2 \cdot 42}{29 \cdot 13.29 \cdot 13} = 18.61.$

$P < 0.01$.

* + = high or moderate bacteriolytic activity; 0 = no lytic activity.

TABLE 3

Fourfold table showing the relation between bacteriolytic action on heat-killed S. aureus and on living S. aureus

LYSIS OF LIVING S. AUREUS	LYSIS OF HEAT-KILLED S. AUREUS		TOTAL
	+	0	
+	26	2	28
0	3	11	14
Total	29	13	42

Chi square $= \frac{[(26.11) - (3.2)]^2 \cdot 42}{28 \cdot 14 \cdot 29 \cdot 13} = 22.28.$

$P < 0.01$.

The bacteriolytic activity of seven-day-old broth-filtrates of the actinomycetes was next studied by the liquid-medium method, using heat-killed and fresh suspensions of both *E. coli* and *S. aureus*. The distribution of lytic properties against heat-killed bacteria was found to be somewhat less common than the distribution of activity associated with the growth of the organisms. On the other hand, lytic activity of filtrates against living *S. aureus* was always slight and was far from common; no action was found on living *E. coli* (table 4). Since indirect evidence has been given that the same lytic agent is responsible for the dissolution of all three susceptible bacterial substrates, the apparent

discrepancy between the action of filtrates on heat-killed or living organisms requires explanation. It may be attributed to quantitative differences, the concentration of the active principle in the filtrates being sufficient to act on the more susceptible heat-killed bacteria but too low to affect the more resistant living organisms. Concentrated preparations of actinomycetin, however, were shown to have no lytic action on living organisms (Welsch, 1941). Another explanation is that the lytic agent acts only on dead bacteria and that actinomycetes able to dissolve living organisms produce not only the lytic principle but also a bacteriotoxic substance. This second hypothesis has been discussed in connection with the study of *Actinomyces* G (Welsch, 1941), and evidence that growth-inhibiting properties of actinomycetes are significantly associated with their ability to lyse living *S. aureus* will be given later.

TABLE 4

Bacteriolytic action of soluble products from actinomycetes

SUSCEPTIBLE ORGANISMS	NUMBER OF FILTRATES OF DIFFERENT ORGANISMS AND THEIR ACTIVITY		
	++*	+	0
Heat-killed *E. coli*	11	18	13
Heat-killed *S. aureus*	11	18	13
Living *S. aureus*	4	0	38
Living *E. coli*	0	0	42

* ++ = highly active; i.e. 25–200 mycolytic units per ml. on heat-killed *E. coli*; turbidity of heat-killed *S. aureus* suspensions reduced to 50% or less of its original value in 2 days or less; turbidity of living *S. aureus* suspension reduced to 75% or less of its original value in 2 days.

\+ = moderately active; i.e. less than 25 mycolytic units per ml. on heat-killed *E. coli*; turbidity of heat-killed *S. aureus* suspension reduced to at most 50% of its original value in 2 days; no action on living *S. aureus*.

0 = no lytic action.

The ability of actinomycetes to manifest "induced antagonism" (Schiller, 1914–1931) was also examined. For this purpose, the strains studied were grown on heat-killed and living suspensions of *E. coli* and *S. aureus*, in nitrogen-free mineral solution, with or without the addition of three per cent sucrose. After incubation at 30° for seven days, transfers from each of the eight cultures obtained with each *Actinomyces* were tested for bacteriolytic action on fresh or killed *E. coli* and *S. aureus*, suspended in nitrogen-free solution and in nitrogen-free soft agar. Transfers from seven-day-old cultures in broth were also used for comparison. In all cases, the activity of every strain tested was highest when grown in nutrient-broth; no "induced" action on *E. coli* was observed, transfers from cultures with bacteria being as inactive as those from broth-cultures. The same negative results were recorded when filtrates from the bacterial suspensions inoculated with the actinomycetes were examined for bacteriolytic activity, in comparison with filtrates from broth cultures.

Although the production of adaptive enzymes by bacteria is a well-known

phenomenon (Dubos, 1940), all attempts to demonstrate the formation of adaptive bacteriolysins by *Actinomyces* G (Welsch, 1941) or by the actinomycetes used in the present study have failed. It may be concluded, therefore, that the theory of induced antagonism cannot be used for an interpretation of the facts observed with these organisms.

The claim of Krassilnikov and Koreniako (1939) and of Kriss (1940) that actinomycetes produce a lytic substance closely related to lysozyme has already been discussed from a theoretical point of view. It was thought desirable to obtain further experimental evidence on the possibility of production of true lysozyme by those organisms. For this purpose, advantage was taken of the high susceptibility of *M. lysodeikticus* (Fleming, 1922) to lysozyme of various origins (Roberts *et al.*, 1938). Suspensions of this organism in mineral solution at 37° were dissolved in a few minutes by a 1/100 dilution of egg-white. Similar suspension, treated with paper-filtrates from four and eight-day-old broth and Czapek's-cultures of the actinomycetes, were not clarified in two days. A reduction of turbidity was observed, however, with four filtrates having a similar action on suspensions of *S. aureus*. This action cannot be attributed to lysozyme since staphylococci are highly resistant to this agent (Thompson and Khorazo, 1935). The particular strain used in these experiments was unaffected after incubation at 37° for two hours in a 1/5 dilution of egg-white.

Suspensions of *M. lysodeikticus* were also inoculated with the different actinomycetes, but lysis was observed only after 48 hours and only with those strains that were able to dissolve fresh *S. aureus* within the same limits of time.

It may, therefore, be concluded that the production of true lysozyme by actinomycetes is yet to be demonstrated.

The distribution of bacteriolytic and bacteriostatic properties among actinomycetes

A study was next made of the distribution of bacteriolytic and bacteriostatic properties among 164 actinomycetes obtained from the New Jersey Type culture collection and of the relations between the two properties. The series of actinomycetes included representatives of the three genera *Actinomyces*, *Proactinomyces*, and *Micromonospora*, and consisted of 62 unidentified strains and of 102 identified organisms, representing at least 85 distinct species or varieties.

Since organisms able to dissolve one of the various susceptible bacterial substrates used are generally capable of acting also on the others, the distribution of bacteriolytic properties among the actinomycetes was studied mainly by the use of living *S. aureus*. This bacterial substrate was previously shown to be the most resistant but also the most interesting.

The three methods of assay above described were used. The final classification of the tested strains into three groups (highly active, moderately active and inactive) was obtained, as shown above.

Broth-filtrates from eight-day-old cultures of the 24 most active organisms and of 43 of the less active forms were tested for lytic action on killed and fresh *E. coli* and *S. aureus*.

As shown in table 5, bacteriolytic properties are widespread among the actinomycetes, but the production of a soluble lytic agent, in a concentration sufficient

to permit detection, appears to be less widely distributed than bacteriolytic ability of the organisms themselves.

Available information on the wide distribution of bacteriostatic properties among soil actinomycetes merely tells us what proportion of active strains may be found in various soils. It does not indicate to what extent antibiotic activities are distributed among the different species or whether these activities are characteristic of certain species or of special strains. According to the state of confusion still existing in the classification of actinomycetes, an answer to those questions is not likely to be soon forthcoming. For practical purposes, the best approach to this problem is probably a systematic study of well-known culture-collection strains. The fungistatic action of 80 different species has already been studied (Alexopoulos, 1941).

Using the cross-streak method above described, it was found that, in addition to its various bacteriolytic abilities, *Actinomyces* G possesses the power of

TABLE 5

Distribution of bacteriolytic properties among actinomycetes

PREPARATION	ACTIVITY ON LIVING S. AUREUS			TOTAL STRAINS
	++	+	0	
Number of strains*	24	54	86	164
Per cent of strains	14.63	32.93	52.44	100
Number of filtrates†	9	11	47	67
	ACTIVITY ON HEAT-KILLED E. COLI AND S. AUREUS			
Number of filtrates†	23	25	19	67

* See table 1.
† See table 4.

inhibiting the growth of several gram-positive bacteria. Inasmuch as such a property has been invoked to explain by a two-step mechanism the bacteriolysis of living organisms (Welsch, 1941), it was thought desirable to study the distribution of bacteriostatic ability among the organisms of the present series, and to compare this distribution to that of bacteriolytic properties.

The bacteriostatic properties of the 164 strains were examined by the cross-streak method. Three test-organisms were used: *E. coli*, *S. aureus*, and *Bacillus subtilis*.

Inhibition of *E. coli* was observed only in one case, namely, by a strain of *Actinomyces lavendulae* studied by Waksman and Woodruff (1942). Inhibition of *S. aureus* was invariably found to accompany inhibition of *B. subtilis*. The zone of inhibition of the coccus, however, was generally smaller than that of the bacillus. *B. subtilis* was inhibited by a number of actinomycetes and the length of the inhibition zone varied from 1 to 40 mm. It was found convenient to classify the organisms on the basis of their inhibitory action upon *B. subtilis*. Thus, actinomycetes producing an inhibition zone of 10 mm. or more were

grouped as highly active; those producing a zone of 1 to 9 mm. were termed moderately active; and a third group comprised all non-inhibiting strains.

Twenty-two highly active organisms, 33 moderately active strains and 12 inactive ones, or 67 strains in all, were tested for the production of soluble antibiotic substances. Tap-water extracts of 10-day-old cultures of these organisms grown on starch-tryptone-agar were obtained, as outlined above. Amounts of 1.0, 0.3, and 0.1 ml. of each filtrate respectively, were added to 10 ml. portions

TABLE 6

Distribution of bacteriostatic properties among actinomycetes

PREPARATION	GROWTH-INHIBITION OF B. SUBTILIS			TOTAL
	++*	+	0	
Number of strains	22	54	88	164
Per cent of strains	13.42	32.93	53.65	100
Number of extracts	5	4	58	67

* ++ = highly active; i.e. inhibition zone 10 mm. long or more; for extracts, inhibition by 0.1 ml. per 10 ml. nutrient agar or less.

\+ = moderately active; i.e. inhibition zone smaller than 10 mm.; for extracts, inhibition by 1 ml. or less, but by more than 0.1 ml. per 10 ml. nutrient agar.

0 = inactive.

TABLE 7

Fourfold table showing the relation between bacteriolytic and bacteriostatic properties of actinomycetes

LYTIC ACTION	BACTERIOSTATIC ACTION		TOTAL
	+*	0	
+	57	21	78
0	19	67	86
Total.	76	88	164

$$\text{Chi square} = \frac{[(57.67) - (19.21)]^2 \cdot 164}{78.86 \cdot 76.88} = 42.76.$$

$P < 0.01$.

* + = high or moderate activity; 0 = no activity.

of melted and cooled nutrient-agar which was then plated and allowed to solidify. Four test-organisms were streaked on each prepared plate, namely, *E. coli*, *S. aureus*, *S. lutea*, and *B. subtilis*. Table 6 shows that bacteriostatic action is widely distributed among actinomycetes, but that only nine out of the 67 filtrates tested, had an antibiotic activity. Filtrates from five species, *Actinomyces antibioticus* (Waksman and Woodruff, 1940), *A. lavendulae* (Waksman and Woodruff, 1942), *Proactinomyces sp.*,[5] *Actinomyces candidus*, and *Micromonospora sp.* were active, inhibition of some of the test-organisms being

[5] This organism was obtained from Dr. Gardner of Oxford.

obtained with less than 0.1 ml. filtrate per 10 ml. medium. Filtrates of four species, namely *Actinomyces roseus*, and three agar-liquefying actinomycetes probably identical with *Actinomyces violaceus* (*ruber*), were weakly active.

No relation was found between the taxonomic position of the organisms and possession of antibacterial properties; in several cases, two or more strains of the same species gave different results. Both bacteriostatic and bacteriolytic properties were found in representatives of the three families, namely, the Actinomycetaceae, Proactinomycetaceae, and Micromonosporaceae. A knowledge of the respective frequency of antibacterial properties in each of these families cannot be obtained at present, since a systematic morphological study of the strains used would be necessary to determine their exact taxonomic position.

Bacteriolytic actinomycetes were found in each of the three groups based on growth-inhibiting-power; however, they were much more numerous among the bacteriostatically active groups. This fact is brought out in the fourfold table (table 7) which, when tested by the "chi-square" method, shows that independence of bacteriolytic and bacteriostatic properties is highly improbable. The fact that bacteriolytic ability is significantly associated with growth-inhibiting power is in good agreement with the two-step theory proposed for the mechanism of lysis of living bacteria (Welsch, 1941).

Action of Actinomyces G and its soluble products on a variety of gram-positive and gram-negative bacteria

The different antibacterial properties previously recognized in *Actinomyces* G are thus shown to be commonly distributed among actinomycetes. Furthermore, the different properties are, most generally, associated in each active organism. Since *Actinomyces* G was in all respects (except for the bacteriostatic action of its filtrate) as active as any other organism studied, it was conveniently taken as a good representative of the kind of activity that may be described as activity of the "actinomycetin type."

Most of the experiments thus far published on the actinomycetin-type of bacteriolysis were conducted with a rather small number of test organisms. In order to find whether the gram-staining properties of the test organisms had a real significance in their susceptibility or resistance (Dubos, 1941), it was thought desirable to examine the action of *Actinomyces* G and its products on a large number of bacteria, heat-killed as well as living.

Altogether, 360 different strains of bacteria were used; they included 100 gram-negative organisms belonging to the genera *Brucella*, *Eberthella*, *Escherichia*, *Erwinia*, *Hemophilus*, *Neisseria*, *Pasteurella*, *Phytomonas*, *Pseudomonas*, *Salmonella* and *Serratia*, as well as 260 gram-positive organisms belonging to the genera *Bacillus*, *Corynebacterium*, *Diplococcus*, *Erysipelothrix*, *Phytomonas*, *Staphylococcus*, and *Streptococcus*.

When heat-killed, all the gram-negative organisms were susceptible not only to the action of growing *Actinomyces* G but also to the action of its active filtrate (actinomycetin); in the latter case, the assay values (Welsch, 1938) obtained by nephelometry, on various suspensions of equal turbidity varied slightly but were

all of the same order of magnitude. On the contrary, all living gram-negative organisms tested were found resistant, not only to actinomycetin but also to the action of the growing *Actinomyces* G. In the latter case, however, some evidence of lysis was observed with species of *Neisseria* and *Hemophilus*, but in both instances it was found that the bacteria were already dead in the suspension medium when the clarification was observed.

All heat-killed gram-positive organisms were more resistant to lysis by growing *Actinomyces* G and by actinomycetin than any of the heat-killed gram-negative ones. In these experiments, when spore-forming organisms were used, care was taken to inactivate the suspension before any appreciable sporulation had occurred.

With living gram-positive organisms, the results obtained were more complex. The action of the growing *Actinomyces* and of actinomycetin should be separately considered, the action on spore-formers and non-spore-formers being different. Growing *Actinomyces* G clarified the suspensions of all gram-positive bacteria studied, including those of spore-bearing organisms made from young, non-sporulated cultures; suspensions made from old cultures consisting mainly of spores, were unaffected. A non-sporulating variant of *B. megatherium* S 36 (den Dooren de Jong, 1931), was dissolved under the same conditions as typically non-sporing bacteria. Actinomycetin reduced the turbidity of the suspensions in mineral-solution of all strains of *Klebsiella pneumoniae* and *Streptococcus hemolyticus* examined. The same action was observed with a few strains of *Staphylococcus aureus* and with *Bacillus megatherium* S 36. With all other organisms suspended in mineral-solution it was impossible to obtain a constant action of the filtrate, although occasional lysis was noted with many of them. Better results were observed with many of the more resistant organisms by transferring sterile fragments of agar-medium from a culture of *Actinomyces* G on a bacterial-agar plate. Although the existence of a bacteriolytic action of actinomycetin on living gram-positive organisms may be considered as certain, the phenomenon is not fully understood as yet.

SUMMARY AND CONCLUSIONS

(1) Bacteriolytic activities against killed bacteria and against living gram-positive bacteria are widely distributed among the actinomycetes. No lytic action was observed, however, against living gram-negative organisms.

(2) Ability to dissolve heat-killed gram-negative and gram-positive bacteria is significantly associated with ability to dissolve living gram-positive organisms. This indicates that the same lytic principle is involved in the action on the three different substrates.

(3) Sterile filtrates of many cultures of actinomycetes examined were able to dissolve heat-killed gram-negative bacteria as well as heat-killed gram-positive organisms. They were, on the contrary, less active, and less frequently so, against living gram-positive bacteria. No lytic action was ever observed against living gram-negative bacteria.

(4) Ability to inhibit the growth of gram-positive bacteria, especially of spore-

bearing organisms, is widely distributed among the actinomycetes. Similar action against gram-negative bacteria was found to be, on the contrary, quite exceptional.

(5) Growth-inhibiting properties appear to be significantly associated with bacteriolytic action upon living gram-positive bacteria.

(6) The group of various antibacterial properties recognized in *Actinomyces* G, which may be designated as the "actinomycetin type" of activity, is commonly found among actinomycetes.

(7) The actinomycetin type of activity was found in representatives of the three genera: *Actinomyces*, *Proactinomyces* and *Micromonospora*. No relation was observed between the taxonomic position of the antagonists and their activity.

(8) No evidence was found for the production of adaptive lysins by actinomycetes grown in association with bacteria.

(9) No evidence was found for the production of true lysozyme by actinomycetes.

(10) The significance of the gram-staining properties in relation to susceptibility or resistance of bacteria to the actinomycetin type of activity was demonstrated by the examination of a large number of strains.

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BIBLIOGRAPHY

Abraham, E. P. 1939 Some properties of egg-white lysozyme. Biochem. J., **33**, 622–630.

Alexopoulos, C. J. 1941 Studies in antibiosis between bacteria and fungi. II. Species of *Actinomyces* inhibiting the growth of *Colletotrichum gloesporioides* Penz. in culture. Ohio J. Sci., **41**, 425–430.

den Dooren de Jong, L. E. 1931 Studien über bakteriophagie. Zentr. Bakt. Parasitenk., I. Orig., **120**, 1–15; 15–23; **122**, 277–286.

Dubos, R. J. 1940 The adaptative production of enzymes by bacteria. Bact. Revs., **4**, 1–16.

Dubos, R. J. 1941 The significance of the structure of the bacterial cell in the problem of antisepsis and chemotherapy. Bicentennial Conferences, University of Pennsylvania Press.

Duché, J. 1934 Les *Actinomyces* du groupe albus. Lechevalier et Fils, Paris.

Epstein, L. A., and Chain, E. 1940 Some observations on the preparation and properties of the substrate of lysozyme. Brit. J. Exptl. Pathol., **21**, 339–355.

Fisher, R. A. 1930 Statistical methods for research workers. Oliver and Boyd, Edinburgh and London, 3d. ed., p. 82, pass.

FLEMING, A. 1922 On a remarkable bacteriolytic agent found in tissues and secretions. Proc. Roy. Soc. (London) B **93**, 306–317.

GASPERINI, G. 1890 Recherches morphologiques et biologiques sur un microorganisme de l'atmosphère, *Streptothrix foersteri* Cohn. Ann. Microgr., **10**, 449–474.

GRATIA, A. 1930–1934 Le traitement des infections à staphylocoque par le bacteriophage et les mycolysats staphylococciques. Bull. mém. soc. nat. chir. Paris., **56**, 344–351; Bruxelles-Méd., *II*. 697–703; Bull. acad. roy. méd. Belg., **14**, 285–300.

GRATIA, A., ET DATH, S. 1924–1931 Propriétés bactériolytiques de certaines moisissures. Compt. rend. soc. biol., **91**, 1442–1443; **92**, 461; 1125–1126; **93**, 451; **94**, 1267–1268; **97**, 1194–1195; **104**, 1058–1059; **106**, 1288–1289.

GRATIA, A., ET RHODES, B. 1924 De l'action lytique des staphylocoques vivants sur les staphylocoques tués. Compt. rend. soc. biol., **90**, 640–642.

KRASSILNIKOV, N. A., AND KORENIAKO, A. I. 1939 The bactericidal substance of the actinomycetes. Microbiology, **8**, 673–685. (Russian with English summary).

KRISS, A. E. 1940 On the lysozyme of actinomycetes. Microbiology, **9**, 32–38 (Russian with English summary).

LIESKE, R. 1921 Morphologie und biologie der Stahlenpilze. Leipzig. p. 130, pass.

MEYER, K., THOMPSON, R., PALMER, J. W., AND KHORAZO, D. 1936 The purification and properties of lysozyme. J. Biol. Chem., **113**, 303–309; 479–485.

NAKHIMOVSKAIA, M. I. 1937 The antagonism between actinomycetes and soil bacteria. Microbiology, **6**, 131–157. (Russian with English summary).

PEARL, R. 1930 Introduction to medical biometry and statistics. Saunders, Philadelphia and London, 2d. ed., p. 317, pass.

ROBERTS, E. A. H. 1937 The preparation and properties of purified egg-white lysozyme. Quart. J. Exptl. Physiol., **27**, 89–98.

ROBERTS, E. A. H., MAEGRAITH, B. G., AND FLOREY, H. W. 1938 A comparison of lysozyme preparations from egg-white, cat and human saliva. Quart. J. Exptl. Physiol. **27**, 381–391.

ROSENTHAL, L. 1925 La lyse des bacilles diphtériques effectuée par un *Streptothrix*. Compt. rend. soc. biol., **93**, 77–79.

SCHILLER, I. 1914–1931 Sur les produits des microbes en association. Zentr. Bakt. Parasitenk., I. Orig., **73**, 123–127; **91**, 68–72; **92**, 124–129; **94**, 64–66; **96**, 54–56; Compt. rend. soc. biol., **91**, 152–153; **96**, 511; **105**, 423–425; **107**, 480–481.

THOMPSON, R. 1941 Certain antibacterial properties of saliva and tears not due to lysozyme. Science, **94**, 77.

THOMPSON, R., AND KHORAZO, D. 1935 Susceptibility to lysozyme of staphylococci. Proc. Soc. Exptl. Biol. Med., **33**, 299–302.

WAKSMAN, S. A. 1941 Antagonistic relations of microorganisms. Bact. Revs., **5**, 231–291.

WAKSMAN, S. A., AND TISCHLER, M. 1942 The chemical nature of actinomycin, an antimicrobial substance produced by *Actinomyces antibioticus*. J. Biol. Chem., **142**, 519–528.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1940a The soil as a source of microorganisms antagonistic to disease-producing bacteria. J. Bact., **40**, 581–600; **42**, 231–249.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1940b Bacteriostatic and bactericidal substances produced by a soil actinomycete. Proc. Soc. Exptl. Biol. Med., **45**, 609–614.

WAKSMAN, S. A., AND WOODRUFF, H. B. 1942 Streptothricin, a new selective bacteriostatic and bactericidal agent, particularly active against gram-negative bacteria. Proc. Soc. Exptl. Biol. Med., **49**, 207–210.

WAKSMAN, S. A., WOODRUFF, H. B., AND HORNING, E. S. 1941 The distribution of antagonistic properties among actinomycetes. J. Bact., **42**, 816 (abstract).

WELSCH, M. 1936–1939 Propriétés bactériolytiques du *Streptothrix* et sporulation. Compt. rend. soc. biol., **123**, 1013–1016; **124**, 573–576; 1240–1242; **125**, 1053–1056; **126**,

247–249; 1254–1256; **127**, 347–349; **128**, 1175–1178; **130**, 104–107; 800–803; **131**, 1296–1299; 3d. Intern. Congr. Microbiol., New York, Rept. Proc. 260–261.

WELSCH, M. 1937 Influence de la nature du milieu de culture sur la production de lysines par les *Actinomyces*. Compt. rend. soc. biol., **126**, 244–246.

WELSCH, M. 1938 La cinétique de la mycolyse. Compt. rend. soc. biol., **128**, 795–801; 1172–1174; **130**, 797–800.

WELSCH, M. 1941 Bactericidal substances from sterile culture media and bacterial cultures, with special reference to the bacteriolytic properties of actinomycetes. J. Bact., **42**, 801–814.

WELSCH, M. 1942 Incidence of bacteriolytic properties among actinomycetes. J. Bact., **43**, 10 (abstract).

tion immediately following sexual fusion instead of a continuance of the heterozygous segregating phase found in higher forms. Under such circumstances only a single heterozygous cell would be formed from each union, it in turn immediately breaking up to the reduced phase on the next cell division thus leading to great difficulty in its detection should the numbers of such fusions be small. This difficulty for the most part can be overcome if the strains tested for sexual fusion differ in several characters.

With strains differing by several characters, differences in the phenotypes of single celled progeny may be observed when mutations take place or when segre-

TABLE 2

Comparative rate of variation of pure stocks and mixed stocks of Phytomonas stewartii grown at 24°C. and plated after 6–7 days' growth

COLONY TUBE NUMBER	STRAIN 509		STRAIN 400		MIXED CULTURE OF STRAINS 509 AND 400		
	Number of colonies observed	Variation	Number of colonies observed	Variation	Number of colonies observed: 509	Number of colonies observed: 400	Variation
1	8,200	No variation	22,400	Pale yellow, 3; rough II, 1	7,260	1,450	No variation
2	11,000	Small raised, mucoid, 4	36,800	Pale yellow, 5	12,200	18,600	Small raised, mucoid, 12; rough II, 2
3	8,400	Small raised, mucoid, 4	11,300	No variation	1,350	5,600	Pale yellow, 3
4	2,800	No variation	14,000	Pale yellow, 2; white, 3	4,900	3,100	No variation
5	11,200	No variation	28,800	Rough III (sector), 1	10,300	19,600	Rough II, 2
6	3,500	Small raised, mucoid, 1	7,600	Rough II, 10	1,650	3,400	No variation
7	5,900	No variation	15,100	Rough II, 7; white, 1	2,200	14,000	Pale yellow, 3
8					1,800	3,000	No variation
9					2,500	4,300	No variation
10					2,300	2,500	Rough mucoid, 1; pale yellow, 1, white, 2

gation occurs if sexual fusion takes place. For the pure parents mutation alone is a source of variation. An estimate of the number of bacteria arising by sexual fusion from such a possible fusion is obtained by contrasting the new forms which arise by mutation in the parent strains with those arising by either cause in the progeny of the mixture of parent types. If the rates for the new forms are equal in both instances the probability of sexual fusion occurring is greatly reduced.

Evidence on this point is presented in table 2. Two parent strains and their mixture were studied using the technique of Lincoln (1940) except that platings were made from cultures more than a day old.

The characteristics of strain 509 were large smooth mucoid white colonies; for

strain 400, small rough non-mucoid dark yellow colonies. Mutations were observed in both the parent cultures and in the co-culture. The range of variation is no greater in the mixed culture than in the parental cultures while the amount of variation in the mixed culture is even lower than the parental cultures. The latter observation is not unexpected since the genetic variability of a mixture of cultures being greater than the variability of a pure culture, fewer variants will be well adapted, continue to reproduce and be observed after the logarithmic phase of growth is passed. Selection in these cultures is so important that no attempt has been made to treat these data statistically.

There is, therefore, no indication of sexual fusion in this species under the conditions of these experiments. This does not mean that sexual fusion may not occur in other species or in this species under other conditions. This latter statement is stressed as the introduction of sexual reproduction may have occurred in other bacterial species in evolutionary history. The methods herein described are general enough to facilitate the investigation of this basic problem in any species.

REFERENCES

LINCOLN, R. E. 1940 Bacterial wilt resistance and genetic host-parasite interactions in maize. J. Agr. Research, **60**, 217–239.

McNEW, GEORGE L. 1938 Dispersion and growth of bacterial cells suspended in agar. Phytopathology, **28**, 387–401.

SHERMAN, J. M., AND WING, H. U. 1937 Attempts to reveal sex in bacteria; with some light on fermentative variability in the coli-aerogenes group. J. Bact., **33**, 315–321.

AGAR-DECOMPOSING STRAINS OF THE ACTINOMYCES COELICOLOR SPECIES-GROUP

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The ability to decompose agar has not hitherto been recorded for any member of the Actinomycetales, and consequently it seemed desirable to make a detailed study of a typical *Actinomyces* possessing this property, which was discovered fortuitously. The organism appeared on a discarded tapwater agar plate (used originally for the germination of myxomycete sclerotia) in the form of blue-black colonies with scanty greyish-white aerial mycelium, each of which lay in the center of a marked depression in the agar. Several strains were isolated and purified by repeated streaking on mineral agar plates with $(NH_4)_2SO_4$ as a nitrogen source. The behavior of the pigment produced (blue under basic, red under acid conditions) marked the organism at once as a member of the widespread soil group of litmus actinomycetes, which are classified in Bergey's Manual under the name *A. coelicolor*.

In response to my request, Dr. S. A. Waksman very kindly provided me with an authentic culture of this species from his collection, together with several agar-decomposing strains which had been isolated at the New Jersey Agricultural Experiment Station in the course of other work. These cultures (NB strains) together with my own isolates (PG strains) comprised the material for the following studies.

MEDIA AND METHODS

The medium used for the maintenance of stock cultures and for the studies on variation consisted of $(NH_4)_2SO_4$ 0.1 g., K_2HPO_4 0.1 g., $MgSO_4$ 0.02 g., NaCl 0.01 g., $CaCl_2$ 0.01 g., $FeCl_3$ 0.002 g., Bacto agar 2.0 g., distilled water 100 ml., pH adjusted to 7.0–7.3. Occasionally the $(NH_4)_2SO_4$ was replaced by KNO_3. The former medium becomes acid, the latter basic, as a consequence of growth, with resultant differences in the color of the pigment, but all other characters of the organisms are identical on both media.

The utilization of carbon sources was studied in liquid media with the mineral base given above (KNO_3 as N-source) and the various substrates to be tested in a concentration of 1.0 per cent.

For the demonstration of agar decomposition Gran's test (flooding the plates with an I-KI solution) was used, although in most cases the gelase fields were already evident without this treatment.

Cultures were incubated at 28°C. In the work on variation, plates were

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always compared and photographed after 4 days at 28°C. followed by 4 days at room temperature, because it was found that an exposure to the lower temperature often resulted in a marked intensification of pigment production and consequent better differentiation of the variant types.

VARIABILITY OF THE AGAR-DECOMPOSING STRAINS

The extreme variability of actinomycetes is a well-known phenomenon, first clearly shown by the able studies of Lieske (1921). However, with the exception of the work of Krassilnikov and Tausson (1938) on *Proactinomyces* species, there has apparently been no detailed investigation since Lieske's time on the occurrence and mechanism of transmissible variations ("Mutationen" *sensu* Lieske) in the mycelial members of this order.

The litmus actinomycetes appear to be an extremely unstable group, a fact attested as early as 1913 by Beijerinck, whose important paper on this subject has been overlooked by most subsequent workers. He wrote of his isolates:

> "Ich fand bei beinahe jeder Aussaat . . . nicht nur verschieden gestaltete Kolonien, sondern auch Sektormutanten, welche bei der Vermehrung entweder atavierten oder erblich stabile morphologische Typen hervorbrachten."

The agar-decomposing strains proved to be no exception to this rule, and since the criterion of agar-decomposition could be used as a check on the origin of the different variants, they provided good material for a re-examination of the subject.

At the time of isolation, a remarkable type of transient biochemical variation was observed. On first streaking the PG strains, only a few agar-decomposing colonies were obtained, interspersed between numerous (*ca.* 95 percent) apparently non-agar-decomposing ones. The former grew far more rapidly than the latter, were more brightly pigmented, and produced an extensive depression of the surrounding agar in 2–3 days. At first it was thought that the cultures contained two different kinds of actinomycetes, but on restreaking, *both types* gave rise to the preceding 95–5 per cent ratio of "non"-agar-decomposers to agar-decomposers. On these plates it was noted that after 7–10 days the "non"-agar-decomposing colonies had begun to attack the agar, although they never attained the size, pigmentation and gelase production of the initially agar-decomposing ones. Again both types were streaked; the "non"-agar-decomposers now began to attack agar in 4–5 days. The same treatment was continued through several more transfers with the ultimate result that the difference in the rate of agar decomposition, and with it the differences in colony aspect, disappeared.

From time to time during this work variant colonies were found which, although they attacked agar readily, differed from the normal type in pigmentation, size, or some other character. One of them (PG 3), a small, very intensely pigmented form with sparse aerial mycelium, yielded a bewildering variety of different types on subculture. Four colonies were selected for further study from among those which appeared on the plate.

1. A small, deeply pigmented[2] colony with no aerial mycelium.

2, 3 and 4. Very irregular colonies, sectored with respect to color and aerial mycelium.

Colony 1 gave an extremely uniform picture on subculture, all the colonies resembling the parent. This type (1a) is illustrated in fig. 2. The strain could be maintained in constant form through repeated subcultures provided that transfers were always made from the substrate mycelium. Type 1a was devoid of aerial mycelium in the younger stages, but an occasional colony would produce a scanty, irregularly sectored aerial mycelium with conidia after about a week, and almost every colony would do so after two or three weeks. If

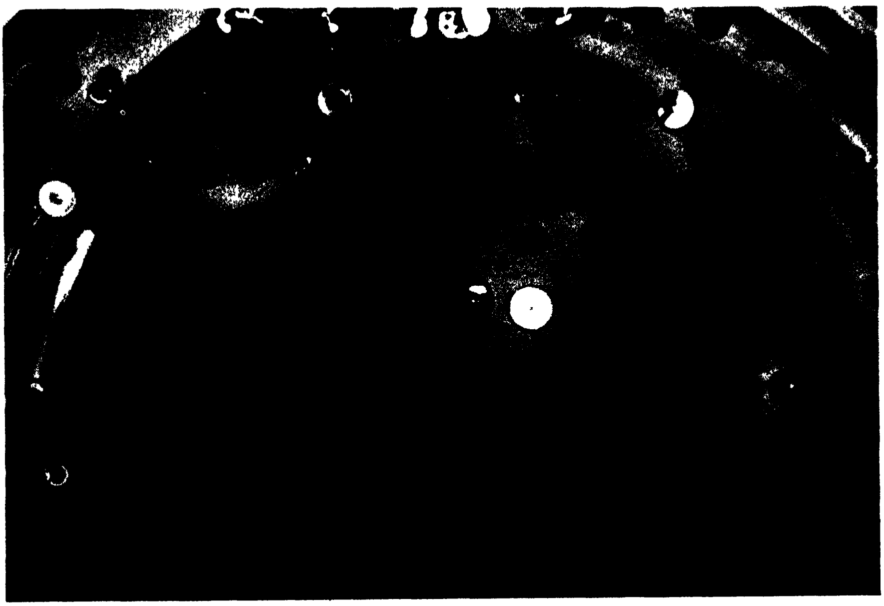

FIG. 1. PLATE CULTURE OF AN UNSTABLE FORM (PROGENY OF A 2B2 TYPE VARIANT), TREATED WITH I-KI SOLUTION TO SHOW THE GELASE FIELDS

All the colonies, despite their extreme dissimilarity, decompose agar. Figs. 2, 3, 4, 5, 7, 8, 10 and 11 are strictly comparable to fig 1 with respect to the age of the cultures when photographed and the magnification of the prints

transfers were made from colonies in this condition an extreme variety resulted, most of the progeny producing conidia in abundance within a few days and bearing not the least resemblance to the parent types (figs. 3 and 4). A further analysis was not attempted.

In the subculture from colony 2 the majority of colonies were large, with an almost colorless or pale yellow substrate mycelium, producing a slight amount of a diffusible yellow pigment and an abundant white aerial mycelium bearing conidia. On ageing, the colonies produced the litmus pigment to a slight degree, the substrate mycelium and surrounding agar becoming green (blue

[2] Except where otherwise stated, pigmentation refers to the litmus-like pigment.

FIG. 2. VARIANT 1A, TREATED WITH I-KI SOLUTION TO SHOW THE GELASE FIELDS
Aerial mycelium begins to become visible on a few colonies

FIG. 3. VARIANT 1A STREAKED FROM THE SUBSTRATE MYCELIUM. COMPARE WITH FIG. 4

litmus pigment plus yellow pigment) or pinkish orange depending on the pH. This type, designated as 2a, proved exceptionally stable through continued transfer, although occasionally one or two deeply pigmented colonies with little aerial mycelium would appear on a plate. Type 2a is illustrated in figs. 5 and 6.

Type 2b, represented by only a few colonies on the original colony 2 plate, was a small, heavily pigmented form with an irregularly sectored grey aerial mycelium. On subculture it gave rise to a large number of further variants, among which stable 2a was common; other well represented types were (1) large heavily pigmented colonies with no aerial mycelium (2b1), (2) typical litmus actinomycete colonies (large and intensely pigmented, the pigment diffusing into the surrounding agar, and with an abundant white aerial mycelium

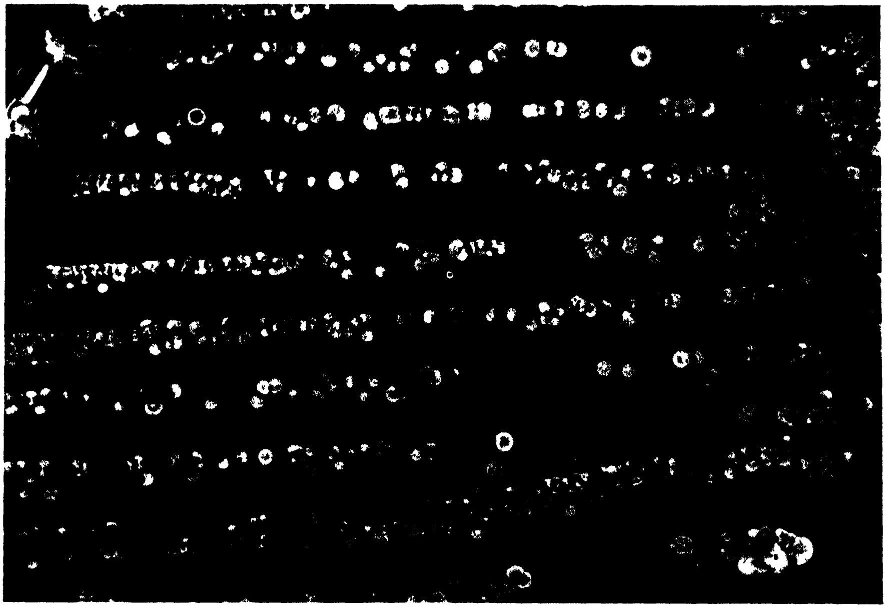

FIG. 1. VARIANT 1A STREAKED FROM CONIDIA

2b2) and (3) colorless, inconspicuous colonies deeply embedded in the agar and without aerial mycelium (2b3). Type 2b1 remained stable on subculture. Type 2b2 was highly unstable, and broke up at once into a large variety of forms among which 2a was common. Type 2b3 remained stable but died out after a few transfers owing to its tendency to undergo a very rapid autolysis. Type 2b3 is represented in fig. 7; figs. 1 and 8 shows examples of the type of subculture resulting from the streaking of 2b2.

Type 2c, like 2b, was represented by only a few colonies on the original plate of 2. The substrate mycelium was colorless or very faintly pigmented and deeply embedded in the agar without at first any aerial mycelium. Older colonies developed a scant aerial mycelium which was always completely devoid of conidia. Type 2c was notable for its remarkable manner of growth, which

was most clearly apparent in well-isolated colonies 3–4 weeks old. The substrate mycelium spread out extensively as an irregular web of hyphae in all

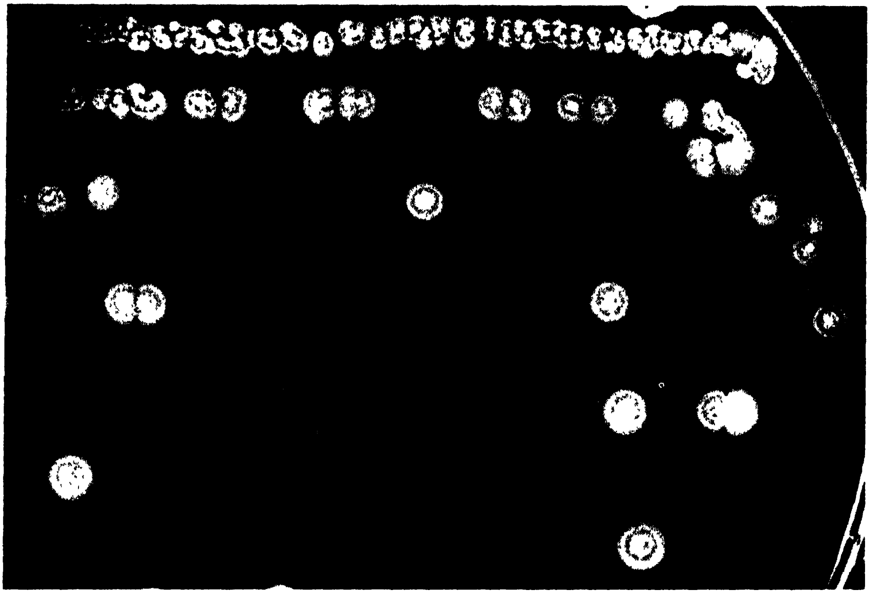

FIG. 5. VARIANT 2A

FIG. 6. PLATE OF VARIANT 2A TREATED WITH I-KI SOLUTION TO SHOW THE GELASE FIELDS

directions below, but not on the surface of the agar. This has never been seen in any of the other variants. Type 2c remained stable on subculture.

FIG. 7. VARIANT 2B3, TREATED WITH I-KI SOLUTION TO SHOW THE GELASE FIELDS
Some of the colonies have begun to autolyze

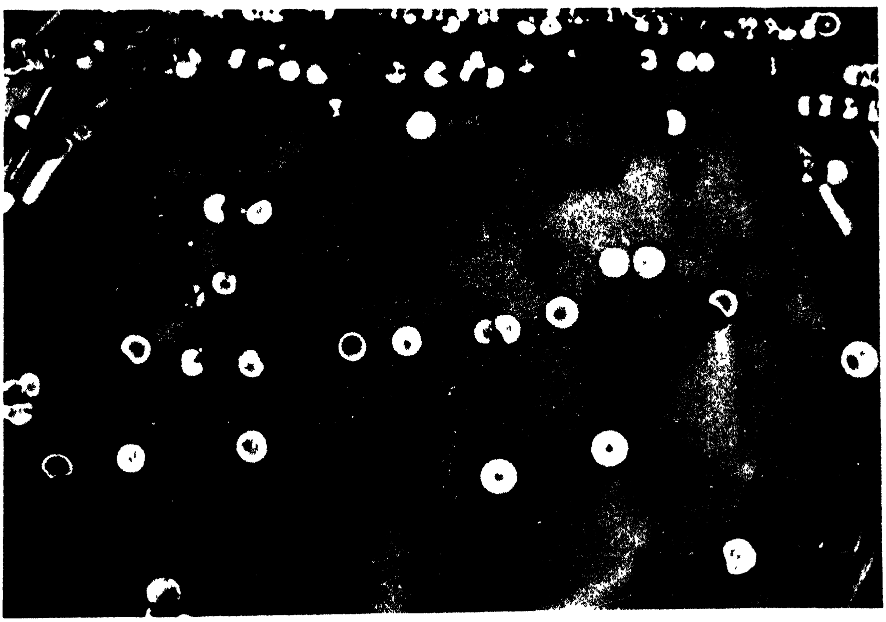

FIG. 8. A TYPICAL EXAMPLE OF THE SUBCULTURES WHICH RESULTED FROM THE STREAKING OF VARIANT 2B2

Colony 3 resulted in a number of variant forms, of which 2a was common and remained, as before, stable on subculture. Type 2b also occurred extensively, giving much the same picture on subculture as previously. In addition a new

FIG. 9. VARIANT 3A. A FEW 2B2 TYPE COLONIES ARE VISIBLE ON THE PLATE

FIG. 10. VARIANT 4A, TREATED WITH I-KI SOLUTION TO SHOW THE GELASE FIELDS

Many of the colonies have already autolyzed, but their sites are visible because of the surrounding gelase fields.

type (3a) appeared, which was characterized by its large maroon colonies either without or with very scanty (conidia-bearing) white aerial mycelium. The maroon pigment appeared to differ from the typical litmus pigment in that it

was retained within the substrate mycelium and never changed color with pH changes. Old cultures produced in addition a small amount of the litmus pigment, which diffused into the surrounding agar. On the whole, 3a was fairly stable but subcultures would split off a few 2b2 colonies, often with considerable sectoring of the aerial mycelium. As before, the 2b2 colonies were

TABLE 1

A comparison of some properties of the variants

TYPE	COLONY SIZE	PIGMENTATION	AERIAL MYCELIUM	CONIDIA	TENDENCY TO AUTOLYSIS	STABILITY
1a	Small	Much litmus pigment	White, scanty, delayed	Present	Absent	Stable except when transferred in conidial stage
2a	Large	Yellow pigment, very little litmus pigment	White, abundant	Present	Absent	Stable
2b	Small	Much litmus pigment	Grey, scanty, sectored	Present	Absent	Very unstable
2b1	Large	Much litmus pigment	None	Absent	Absent	Stable
2b2	Large	Much litmus pigment	White, abundant, often sectored	Present	Absent	Very unstable
2b3	Small	None	None	Absent	Considerable	Stable
2c	Large	Very small amount of litmus pigment	Scanty, delayed	Absent	Absent	Stable
3a	Large	Much maroon pigment, a little litmus pigment	Scanty, delayed	Present	Absent	Fairly stable
4a	Very small	None	None	Absent	Considerable	Stable

highly unstable, giving rise to a number of different types, predominant among which was 2a. 3a is illustrated in fig. 9.

Colony 4 behaved similarly to 3. However, in addition to the variants found on 3, there appeared a few extremely small, colorless, inconspicuous colonies without any aerial mycelium which decomposed agar only to a very slight extent (4a). This type maintained its characters on subculture, but died out after only a few transfers due to its tendency to rapid autolysis (fig. 10). Except

for the difference in size and gelase fields (compare figs. 7 and 10), 4a was very similar to 2b3. In table 1 some properties of the variants have been summarized and compared.

NUTRIENT REQUIREMENTS AND CULTURAL CHARACTERISTICS OF THE DIFFERENT STRAINS

The following nine strains were used: NB 3442, NB 3443, NB 3445, NB 3355 (authentic non-agar-decomposing culture of *A. coelicolor*), PG 1, PG2, PG3 variant 1a, PG 3 variant 2a, PG 3 variant 3a.

The availability of different carbon sources as judged by growth in their presence showed a remarkable general uniformity. Of the simple sugars and polyalcohols, glucose, galactose, maltose, lactose, cellobiose, glycerol and mannitol supported excellent growth in all cases. On the other hand, none of the strains attacked xylose, arabinose or levulose after 10 days, although later a very slight amount of growth on these sugars occurred with some strains. The growth with sucrose was generally weak, only PG 3 variant 1 showing good development. Agar and alginic acid were readily attacked except by NB 3355, which was unable to grow at all on them. Cellulose (in the form of cellophane strips) was utilized in all cases. All strains grew well on succinate and malate but were unable to use acetate, citrate and lactate, with the exception of NB 3355, which developed satisfactorily on lactate.

It is thus apparent that a definite general pattern of carbon utilization runs through the whole group. NB 3355, the authentic *A. coelicolor*, is the only strain which diverges markedly from the general pattern through its inability to attack agar and alginic acid and its good growth with lactate. The three variants of PG 3 which were included gave results differing in no essential respect from those obtained with the other agar-decomposing strains.

The cultural studies on these nine strains showed once again the worthlessness of criteria such as pigmentation on different media and proteolytic activity now commonly used in the differentiation of *Actinomyces* species. In spite of the fact that all strains (with the possible exception of NB 3355) were undoubtedly closely related, there were in many cases marked cultural divergencies between them, which stood out in contrast with the general uniformity exhibited in the utilization of different carbon sources. Furthermore, in a number of cases there were considerable differences between cultures of a single strain grown on Czapek's agar, depending on the temperature of incubation. For these reasons it seems unnecessary to give details of the results.

The only reliable distinction between the authentic culture of *A. coelicolor* and the agar-decomposing strains would appear to be the inability of the former to attack agar and alginic acid, which can hardly be considered sufficient reason for a specific separation.

DISCUSSION AND POSSIBLE INTERPRETATIONS OF THE VARIATION PHENOMENA

Let us consider first the biochemical variation exhibited by the PG strains during the early stages of their cultivation. The 95–5 ratio of apparently non-

agar-decomposing to agar-decomposing colonies occurring irrespective of which type was used as inoculum, together with the fact that the apparently non-agar-decomposing type would produce the enzyme gelase after a considerable time lapse, indicate the common origin of both types. Conidia were always used in making transfers, so that each successive generation developed through the conidial stage. The conclusion is thus unavoidable that the process of conidium formation was in some way responsible for the observed phenomena.

The constant ratio maintained between the two biochemical types on subculture from either one is suggestively similar to the quantitative relationships reported by Bunting (1940) in her studies on color variations in *Serratia marcescens*, except that in the latter case the constant ratios were obtained only gradually over several transfers. It is possible that the same sort of mechanism operates in both cases, but my data are insufficient for a strict comparison.

Since agar was the sole source of carbon and energy—apart from incidental impurities—in the medium employed, it would seem reasonable to assume that the differences in colony size, pigmentation, etc., between the two types were ultimately due to a difference in the rate of gelase production. This assumption gains further support from the fact that the elimination of the difference in the rate of gelase production which resulted from repeated transfer on the same medium also eliminated the other differences.

At first it seemed that such behavior could be explained on the assumption that gelase was an adaptive enzyme in the slow agar-decomposing colonies and constitutive in the rapid agar-decomposing ones. On this hypothesis, however, one ought to find that continued cultivation in a medium devoid of agar would result in a loss of the ability to produce gelase immediately on return to an agar medium for at least a considerable proportion of the organisms. Such was not the case; several cultures which had attained a uniform rate of agar decomposition were carried through a series of transfers over a period of two months in liquid media containing glucose, galactose or glycerol as carbon sources, without the slightest diminution in the rate of gelase production being apparent on their return to agar.

The task of attempting an interpretation of the variations in colony form is equally difficult. For several reasons it does not seem possible to fit these observations into the well-known dissociative pattern of bacteria. The number of sharply distinct variant types was far greater than the number to be expected on the basis of M-S-R changes, and there were very few types which could be regarded as intermediate (i.e., SR) forms. Furthermore, there was no very obvious regularity or predictability about the changes observed—the more unstable variants gave rise at once on transfer to a wide variety of totally dissimilar types instead of to the chief dissociative form and intermediates which one normally finds in bacterial dissociation. It must be admitted, however, that the number of variable characters studied simultaneously in this case is far greater than with most bacteria.

A survey of the results leads to a few tentative conclusions as to the *probable* changes which may occur. Outstanding in this connection is the effect of

conidium formation. Colony types which can be transferred in the mycelial stage—either because the formation of conidia-bearing aerial hyphae is delayed, or because the aerial hyphae are devoid of conidia, or because an aerial mycelium is never formed—are likely to remain stable. This contention is supported by the behavior of types 1a, 2b1, 2b3, 2c and 4a. That their stability may be ascribed to the lack of conidium formation is shown by the results obtained when transfers of 1a were made after an aerial mycelium had developed. On the other hand, variants producing an aerial mycelium with conidia are not necessarily unstable; this is outstandingly exemplified by type 2a, which, in spite of the fact that it always produced conidia in abundance, remained the most consistently stable of all the variants.

Thus my observations would seem to indicate that it is primarily the process of conidium formation which is responsible for the great variability of the *Actinomyces* strains studied. That other mechanisms of variation, operative through a non-conidial phase of growth, are to be found in the Actinomycetales, has been shown beyond doubt by the work of Krassilnikov and Tausson (1938) on *Proactinomyces* species. It may be that the phenomena are homologous, but at present we do not have sufficient evidence to form an opinion on that point. Consequently the following discussion of possible mechanisms is limited to the conidial type of variation.

The cytological findings of Badian (1936) are of considerable interest in connection with the present work, since they provide a possible basis for explaining conidial variation. Badian claimed that the chromatin material of actinomycetes is distributed through the hyphae in the form of chromosome-like bodies which undergo autogamy just before conidium formation, with the result that each conidium contains a bivalent chromosome. When the conidium germinates, this bivalent chromosome undergoes two divisions, one of them a reduction division. At the same time, from one to three germ tubes are formed, each of which receives one of the four daughter chromosomes. The remaining chromosome or chromosomes suffer a gradual degeneration. This work has, it is true, been sharply criticized by Schaede (1939) and von Plotho (1940). However, the negative results obtained by these investigators cannot logically be advanced to disprove Badian's *observations*, supported as these are with excellent photomicrographs. The sole explanation offered by Schaede, that the bodies which Badian took for chromosomes were actually condensed cytoplasm, is not a convincing one. Furthermore, cytological studies by Badian on myxobacteria (1930, 1933) and on yeasts (1937) in which he used the same technique as for his actinomycete studies, have checked to a considerable extent with those of Beebe (1941) on myxobacteria and Rochlin (1933) on yeasts in which different techniques were employed. For these reasons it would seem premature to regard his observations either as figments of the imagination or as artefacts resulting from the use of faulty methods.

Badian has used his own observations to explain the phenomenon of sector formation in actinomycetes as follows:

"Zytologische Erscheinungen, wie sie oben beschrieben wurden, machen es nun wahrscheinlich dass Sektorenbildung und Mendelsche Spaltung wirklich wesensverwandt sind. Da das Chromosom der Spore bivalent ist und zwei univalenten entspricht, die sich autogamisch vereinigt haben, so muss es nicht unbedingt immer homozygotisch sein. Ist es aber heterozygotisch, so werden während der Chromatinreduktion zwei Chromosome das eine, die zwei anderen aber das andere Gen von einem Allelomorphenpaar erhalten. Wachsen nun solch einer Spore drei Keimschläuche hervor, so müssen zwei von ihnen gleichartige Chromosome enthalten und darum erbgleich sein, der dritte wird aber ein im genetischen Sinne abweichendes Chromosom aufweisen. Wachsen nun die Keimschläuche aus, so wird die Zone, die aus Verzweigungen dieses dritten Fadens besteht, sich als ein Sektor mit abweichenden Eigenschaften hervorheben."

Of course this explanation covers only those cases where a single sector is formed, not examples (like that illustrated in fig. 11) of multiple sector formation. However, it is quite possible that multiple sectored colonies have developed from more than one conidium, in which case Badian's explanation would still remain valid. Furthermore, by assuming that in many cases only

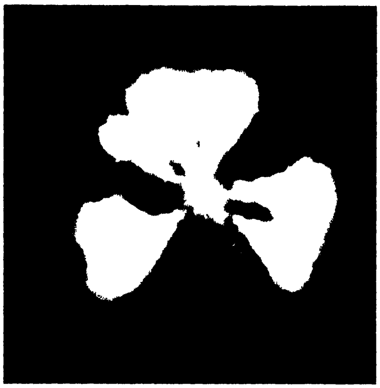

Fig. 11. A Typical Example of Multiple Sector Formation

one or *two* germ tubes grow out from a germinating conidium, one can use Badian's hypothesis to explain that marked heterogeneity so often characteristic of the progeny of unstable strains. Colonies developing from a single germ tube will (if we accept Badian's explanations) always be homozygous, while at least 30 per cent of those developing from two germ tubes will be so. Thus a heterozygous condition in an *Actinomyces* colony would be expressed in the appearance of separate and distinct colony types if transferred through the conidial stage, but not if transferred in mycelial state. Cases of extremely stable strains producing an abundance of conidia (e.g., 2a) can also be explained on the assumption that such strains are completely homozygous. This is not to be taken as more than a mere working hypothesis, wholly dependent as it is on the outcome of future cytological work.

SOME REMARKS ON THE TAXONOMY OF ACTINOMYCES SPECIES

Although the limits of the genus *Actinomyces* are fairly clear, species differentiation is in a most unsatisfactory state. Because of the proven unreli-

ability of nearly all the differential characters which have been suggested or used, it has seemed to many that the attempt to erect valid species is foredoomed to failure. The most notable example of this pessimism is to be found in Lieske (1921) who, after years of work with these forms, rejected the possibility of a satisfactory systematic treatment.

Since Lieske's time there has been little published which would justify a more optimistic outlook. The painstaking morphological work of Ørskov (1923) laid the foundations for a better understanding of fundamental morphology in the Actinomycetales as a whole, but provided no help for species differentiation. More recently, Waksman (1940) has recognized five sub-groups in the genus *Actinomyces* on the basis of the structure of the sporulating hyphae, but this treatment provides at best only a partial solution to the problem, since in each of these sub-groups there are a number of species which must ultimately be differentiated by other means.

Conn and Conn (1941) have made what promises to be an extremely important contribution from the physiological aspect through their attempt to re-evaluate the character of chromogenesis. They have stressed the fact (which, as they say, has long been an open secret among students of the group) that many actinomycete pigments act as hydrogen ion indicators, and they have pointed out that the nature of the pigment, rather than the color produced, is the important character. The case of the litmus actinomycetes affords a striking demonstration of their contention. Actually the behavior of the pigment in this group was clearly described by Beijerinck in 1913; nevertheless, even this outstanding example of an indicator pigment has been overlooked or misunderstood. In the fifth edition of Bergey's Manual no mention is made of it in the description of *A. coelicolor*, although hints are provided for the observant taxonomist in the key:

1. Pigment blue, not always definite.
   b. Soluble red pigment, turning blue on synthetic (sic) agar.
   52. *Actinomyces coelicolor.*

One point of some importance has gone unmentioned by the Conns; namely, the possibility that more than one pigment may be produced by an organism. In the strains which have been the object of the present study, a pale yellow pigment is often produced.[3] It is not observable in cultures producing an abundance of the litmus pigment, but when the latter is present in only slight amounts the yellow pigment often causes cultures to appear green. This shows that in order to use the pigmentation as a differential character it will be necessary not merely to record the pH of the medium but to make an extraction of the culture, a separation of the pigment components and a study of the behavior of each one alone. Such studies must be carried out with a variety of media, since on certain substrates the production of the characteristic pigment may be slight or even absent. It remains to be seen in how far there

[3] This was also noted by Beijerinck (1913) in the strains studied by him.

occurs a *permanent* loss of the ability to produce pigments of special types; the Conns consider this character to be stable, but there are some indications from my work that unpigmented variants (e.g., 2b3 and 4a) can be derived from litmus actinomycetes.

Finally, I should like to draw attention to the possibility that the utilization of different carbon sources may prove a desirable taxonomic criterion. It is true that attempts to use a *single* character of this type (e.g., the ability to decompose cellulose) as was done by Krainsky (1914) have not been successful, but the *general pattern* of carbon utilization in actinomycetes has never been subjected to a systematic investigation. The remarkable uniformity with respect to carbon sources in the strains I have studied suggests that in future this may be a profitable approach. Since the majority of soil actinomycetes will grow in a medium with mineral nitrogen alone, the experimental technique adopted by den Dooren de Jong (1928), which makes easy the simultaneous testing of a large number of strains on a wide variety of carbon sources, could in most cases be employed to advantage.

In conclusion, I should like to express my thanks to Dr. C. B. van Niel and to Dr. A. T. Henrici for their valued criticism and advice.

SUMMARY

Attention is drawn to the existence, previously unrecorded, of agar-decomposing organisms among the Actinomycetales. Several strains have been studied and shown to belong to the *Actinomyces coelicolor* species-group.

On first isolation several of these strains gave rise to colonies which varied in their ability to attack agar, but on continued cultivation the differences in this respect disappeared. During the subsequent work marked variations in colony form, conidium formation, pigmentation, and other characters were noted. Some of these variants were stable, whereas others continuously gave rise to new types.

An analysis and interpretation of the possible mechanisms of variation in these organisms has been attempted.

REFERENCES

Badian, J. 1930 Zur Zytologie der Myxobakterien. Acta Soc. Botan. Poloniae, **7**, 55–71.

Badian, J. 1933 Eine zytologische Untersuchung über die Myxobakterie *Polyangium fuscum.*, Acta Soc. Botan. Poloniae, **10**, 361–378.

Badian, J. 1936 Ueber die zytologische Struktur und den Entwicklungszyklus der Actinomyceten. Acta Soc. Botan. Poloniae, **13**, 105–126.

Badian, J. 1937 Sur la cytologie des levures. Bull. intern. acad. polon. sci., Classe sci. math. nat., B, I, 61–87.

Beebe, J. M. 1941 The morphology and cytology of *Myxococcus xanthus*, n. sp. J. Bact., **42**, 193–224.

Beijerinck, M. W. 1913 Ueber Schröter und Cohn's Lakmusmicrococcus. Folia Microbiol., **2**, 185–200. (Reprinted in Beijerinck's Verzamelde Geschriften, vol. 5, 149–160.)

BUNTING, M. I. 1940 The production of stable populations of color variants of *Serratia marcescens* 274 in rapidly growing cultures. J. Bact., **40**, 69–81.

CONN, H. J. AND CONN, J. E. 1941 Value of pigmentation in classifying actinomycetes. A preliminary note. J. Bact., **42**, 791–800.

DEN DOOREN DE JONG, L. E. 1926 Bijdrage tot de kennis van het mineralisatieproces. Thesis, Delft.

KRAINSKY, A. 1914 Die Aktinomyceten und ihre Bedeutung in der Natur. Zentr. Bakt. Parasitenk., II, **41**, 649–688.

KRASSILNIKOV, N. A., AND TAUSSON, T. A. 1938 Variability of proactinomycetes and mycobacteria. Microbiology, **7**, 50–74.

LIESKE, R. 1921 Morphologie und Biologie der Strahlenpilze. Leipzig, Gebr. Borntraeger.

ØRSKOV, J. 1923 Investigations into the morphology of the ray fungi. Copenhagen, Levin and Munksgaard.

VON PLOTHO, O. 1940 Die chromatische Substanz bei Actinomyceten. Arch. Mikrobiol., **11**, 285–311.

ROCHLIN, E. 1933 Ueber die Nuklealreaktion bei Hefen. Zentr. Bakt. Parasitenk., II, **88**, 304–306.

SCHAEDE, R. 1939 Zum Problem des Vorkommens von chromatischer Substanz bei Bakterien und Actinomyceten. Arch. Mikrobiol., **10**, 473–507.

WAKSMAN, S. A. 1940 On the classification of actinomycetes. J. Bact., **39**, 549–558.

CLASSIFICATION OF 110 STRAINS OF STAPHYLOCOCCUS AUREUS

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A collection of strains of *Staphylococcus aureus* was studied with a view to finding out what relationship, if any, could be detected between the agglutinative grouping and the production of polysaccharide, alpha hemolysin, or leucocidin by the various strains.

A number of serological groupings for staphylococci have been described in the literature, the most recent ones being those of Blair and Hallman (1936), Yonemura (1936), Cowan (1938), and Christie and Keough (1940). The classification put forward by Cowan has probably been the most widely adopted, and is the one employed in this study. Of the polysaccharide classifications, the "A" and "B" classification of Julianelle and Wieghard (1935) remains fundamental for pathogenic staphylococci. Thompson and Khorazo (1937) have found three additional groups containing mostly non-pathogenic staphylococci, and Cowan (1938) places pathogenic strains giving irregular polysaccharide reactions into a general class "C". The Julianelle classification is the one employed for the work described in this paper.

A staphylococcal leucocidin which destroys rabbit leucocytes was described by Van der Velde (1894) and studied further by Neisser and Wechsberg (1901). More recently (1936) Wright has worked with this Neisser-Wechsberg leucocidin, and she suggests, as do also Valentine (1936) and Proom (1937), that it is identical with staphylococcal alpha-hemolysin.

Panton and Valentine (1932), Valentine (1936), and Valentine and Butler (1939), however, have demonstrated that certain strains of staphylococci elaborate a leucocidin which is distinct from the Neisser-Wechsberg type. The Panton-Valentine leucocidin is active against human as well as rabbit leucocytes, thus differing from the Neisser-Wechsberg leucocidin, which does not affect human leucocytes. The two leucocidins also differ in their mode of attack upon the leucocytes. Both cause disintegration of the cell nucleus, but the appearance of the damaged particles is different. Panton-Valentine leucocidin in stronger concentration produces complete disintegration of the cytoplasm. Panton-Valentine leucocidin is the one referred to in this paper.

Dr. John E. Blair of the Hospital for Joint Diseases, New York, supplied us with Cowan group I and group III strains, and Dr. Champ Lyons of Harvard Medical School, the group II strain. Dr. L. A. Julianelle of Washington University School of Medicine, Saint Louis, sent us his representative polysaccharide "A" and "B" strains.

All the cultures included in the study were isolated from human infections. The origin of the strains is shown in table 1.

METHODS AND PROCEDURES

Agglutinating sera were prepared from the three Cowan group strains and the cultures under study were classified by slide agglutination. The sera were prepared by immunizing rabbits with organisms from 18-hour beef-infusion broth cultures. The growths were resuspended in physiological salt solution containing 0.5 per cent formalin. The group II serum as prepared, contained considerable group III agglutinin, which was absorbed out. No preliminary absorption was necessary with the antisera of the other groups.

For antigens intended for the method of slide agglutination, 3-hour growths were used, as recommended by Cowan (1939). It was found that the employment of the trypsin-digest beef-heart broth described by Pauli and Coburn (1937) for streptococci, greatly reduced the incidence of granular growth or autoagglutination, and this broth was used to grow the antigens. The antigens were heated rapidly to boiling according to Cowan's method, centrifuged, and resuspended in trypsinized broth, with a density of 100 billion organisms per milliliter.

TABLE 1

Origin of strains

Infection of skin	29
Infections of bone	16
Septicemia and bacteremia	17
Miscellaneous internal infections	7
Type of lesion not stated in history	41

For the tests, 0.01 ml. of antigen and 0.03 ml. of serum (1–5 dilution) were mixed on a glass plate and rocked 5 minutes. Readings were made with a hand lens against an illuminated dark background.

Extracts for carbohydrate detection were prepared from 103 strains of the series by the method of Julianelle and Wieghard (1935). A uniform volume of broth, 600 ml., was employed in all cases, and the extract obtained was made up to a volume of 60 ml. The extracts were tested against sera produced in rabbits with the Julianelle "A" strain 13 and the "B" strain M11.

Forty-eight of the strains studied were tested for alpha-hemolysin production. Cultures were grown 48 hours in veal-infusion, semi-solid agar in an atmosphere of 80 per cent oxygen and 20 per cent carbon dioxide. The fluid obtained from the medium was Berkefeld-filtered and tested for toxin with a 1 per cent suspension of rabbit erythrocytes. Strains that had been carried in the laboratory for a number of years were not tested for hemolysin-production.

Recently isolated strains were tested for leucocidin production. The strains were grown on veal-infusion agar, according to the method described by Valentine (1936), and tested for potency by his method, with human leucocytes.

RESULTS

When tested against the Cowan sera, the cultures were divided as shown in table 2.

Several of the negative strains showed a common antigen not contained in any of the Cowan groups, and these apparently constitute a supplementary group. The type strain of this group, no. 235 (Smith strain) of the collection, was received by us from Dr. René J. Dubos of the Rockefeller Institute and had been obtained from a human infection.

The relationship between the agglutinative grouping and the polysaccharide reactions of the strains is shown in table 3.

As indicated in table 3, extracts of 85 strains gave strongly positive reactions for Julianelle "A" polysaccharide, while 18 reacted weakly or not at all. None of the extracts gave "B" polysaccharide reactions. All strains produced coagulase except two members of the "ungrouped" negative agglutinative group. Another member of the negative group was the sole non-fermenter of mannitol. The three strains that gave these irregular reactions did not produce detectable "A" polysaccharide.

TABLE 2

Agglutinative groups

Group I	38 strains
Group II	10 strains
Group III	36 strains
Group I or III	17 strains
Negative (ungrouped)	9 strains

TABLE 3

Agglutinative and polysaccharide relationships

COWAN AGGLUTINATIVE GROUP	JULIANELLE "A" POLYSACCHARIDE		JULIANELLE "B" POLYSACCHARIDE	
	Positive	Slight or negative	Positive	Negative
I	29	2	0	31
II	6	4	0	10
III	31	4	0	35
I or III	16	1	0	17
235 group	2	1	0	3
Ungrouped	1	6	0	7

Table 3 shows that of 10 group II strains, 4 or 40 per cent yielded a lessened amount of "A" polysaccharide, but of 93 strains exclusive of group II, 14 or only 15 per cent yielded a lessened amount of "A" polysaccharide. This is in agreement with the observation of Cowan (1939), that irregularities of polysaccharide production are most frequently associated with group II.

All our recently isolated group II strains were leucocidin producers. It was considered useless to test old strains for leucocidin production. Most of the leucocidin-producing strains belonged to group II, and the majority of them appeared to produce less than the average amount of "A" polysaccharide. Of 4 members of group III that were weak in "A" polysaccharide, two were leucocidin

strains. Of the total number of cultures examined, 10 per cent belonged to group II, but of leucocidin-producing strains, 55 per cent belonged to group II.

Of the total number of cultures examined, 17 per cent produced a lessened amount of "A" polysaccharide, but of leucocidin-producing strains, 66 per cent produced a lessened amount of "A" polysaccharide.

It is hardly safe to generalize from so small a number of cultures, but so far as this series is concerned, it appears that leucocidin producers frequently fall into the Cowan II group, and that leucocidin-producing strains show a tendency to produce less Julianelle "A" polysaccharide than other strains.

It is not the intention to suggest that these strains are entirely devoid of "A" polysaccharide. If a more concentrated extract were prepared, perhaps all of them would show a strongly positive reaction. However, when carbohydrates were prepared from all the strains studied, employing a uniform volume of broth and a uniform volume of final product, the leucocidin-positive strains referred to,

TABLE 4

Slide-agglutination with Cowan and 235 sera

Cowan grouping

	GROUP I SERUM (S11)	GROUP II SERUM (S80)	GROUP III SERUM (S33)	235 SERUM
S11	++++	+	+	+
198	++++	+	++++	++++
S80	+	+++	+	−
S33	+	−	++++	+
235	−	−	−	++++
114	−	+	−	++++
207	−	−	−	++++

appeared to be weaker as "A" carbohydrate producers than the majority of strains in the series.

No agglutinative group seemed to be associated with any particular type of infection, but on the contrary, strains obtained from different lesions were distributed indiscriminately. Alpha-hemolysin producers occurred among members of all the groups.

Strain 235 produces a very weak alpha-hemolysin and no leucocidin, but is unusually pathogenic for mice. It gives an entirely negative reaction to sera of the three Cowan groups, but is strongly positive to its homologous serum.

Table 4 shows the relationship between the Cowan strains S11 (group I), S80 (group II) and S33 (group III) and strains related to 235.

It will be noted that certain strains show a strong affinity for the serum prepared with strain 235 and are non-reactive toward the Cowan sera. The Cowan group strains, on the other hand, show but slight reactivity toward the 235 serum.

Agglutinin absorption tests were run using the Cowan and 235 sera and cultures, and the absorbed sera were tested against the strains shown in table 4.

Each serum was absorbed with a suspension containing 200 billion organisms per ml. The dilutions of the absorbed sera were made to one tenth of the maximum titer which could be obtained with homologous organisms.

TABLE 5

Agglutinin absorptions of group sera

ANTIGEN	S11	S80	S33	235	CONTROL, UNABSORBED SERUM
Absorption of group I serum					
S11		1:5	1:5	1:5	1:50
198		1:5			1:500
S80					1:25
S33		1:5			1:5
235					
207					
114					
Absorption of group II serum					
S80				1:5	1:50
114	1:5	1:25	1:5		1:50
S11					1:5
198		1:5			1:50
S33					1:5
235					
207					
Absorption of group III serum					
S33					1:50
S11				1:5	1:25
198		1:5			1:50
S80	1:5				1:25
114					
235					
207					
Absorption of 235 serum					
235	1:25	1:25	1:25		1:50
207		1:100			1:1000
114	1:5	1:50	1:5		1:50
198		1:50			1:50
S11					
S80					
S33					

Results are shown in table 5.

It appears from table 5 that strains 235 and 207 are nearly identical in antigenic consitution and but slightly related to the Cowan groups, while strain 114

contains some group II antigenic substance. However, strain S80 (group II) is only partially able to absorb agglutinins for 114 from the sera. Strain 198 reacts as a group I or group III strain that contains some 235 antigenic substance. Strain 198 is the Gamma-hemolysin strain of Llewellyn Smith (1938) and, like 235, is unusually pathogenic for mice. S33, when used in anounts of 200 billion organisms per milliliter, absorbs out nearly all agglutinins from the sera, so far as is shown by slide-agglutinative tests. Cowan (1939) warns of this tendency of S33 to become non-specific, and advises frequent passages through rabbits to maintain its specificity. Mice were employed instead of rabbits for this study. The S33 culture was passed through mice frequently and the cultures for use were inoculated from the blood, or in some cases the blood cultures were stored in vacuo until used. In spite of this treatment S33 showed considerable non-specific reactivity.

SUMMARY

The agglutinative and polysaccharide-forming qualities of a collection of *Staphylococcus aureus* strains were studied. Most of the strains belonged to the Cowan groups I and III and produced Julianelle "A" polysaccharide. Leucocidin-positive strains belonged mainly to group II. Most leucocidin-positive strains showed a tendency to produce less "A" polysaccharide than other strains.

A supplementary agglutinative group is described in addition to the three Cowan groups.

REFERENCES

Blair, J. E., and Hallman, F. A. 1936 Serologic grouping of Staphylococci. J. Bact., **31**, 81.

Christie, R., and Keough, E. V. 1940 Physiological and serological characteristics of staphylococci of human origin. J. Path. Bact., **51**, 189–197.

Cowan, S. T. 1938 The classification of staphylococci by precipitation and biological reactions. J. Path. Bact., **40**, 31–45.

Cowan, S. T. 1939 Classification of staphylococci by slide agglutinations. J. Path. Bact., **48**, 169–173.

Julianelle, L. A., and Wieghard, C. W. 1935 The immunological specificity of staphylococci. J. Exptl. Med., **62**, 11–21.

Neisser, M., and Wechsberg, F. 1901 Über das Staphylotoxin. Z. Hyg. Infektionskrankh., **36**, 299–349.

Panton, P. N., and Valentine, F. C. O. 1932 Staphylococcal Toxin. Lancet, **222**, 506–508.

Pauli, R. H., and Coburn, A. F. 1937 Studies on the serological typing of streptococcus hemolyticus. J. Exptl. Med., **65**, 595–612.

Proom, H. 1937 The interrelationships of staphylococcal leucocidins. J. Path. Bact., **44**, 425–429.

Smith, M. L., and Price, S. A. 1938 Staphylococcus gamma hemolysin. J. Path. Bact., **47**, 379–393.

Thompson, R., and Khorazo, D. 1937 Correlated antigenic and biochemical properties of staphylococci. J. Bact., **34**, 69–79.

Valentine, F. C. O. 1936 Further observations on the role of the toxin in staphylococcal infection. Lancet, **230**, 526–531.

Valentine, F. C. O., and Butler, E. C. B. 1939 Specific immunity in acute staphylococcal osteomyelitis. Lancet, **236**, 973–978.

Van der Velde, H. 1894 Etude sur le mécanisme de la virulence du staphylocoque pyogène. La Cellule, **10**, 401.

Wright, J. 1936 Staphylococcal leucocidin (Neisser-Wechsberg type) and antileucocidin. Lancet, **230**, 1002–1004.

Yonemura, N. 1936 Über die immunisatorische Einteilung von Staphylococcus pyogenes. Z. Immunitäts., **89**, 392–397.

ULTRACENTRIFUGATION AND CYTOLOGY OF SPIRILLUM VOLUTANS[1]

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During the past few years the authors have been interested in studying the effects of high centrifugal force upon the structure of various types of cells in both plants and animals (Beams and King, 1940). In the course of these investigations a large flagellated bacterium, *Spirillum volutans*, appeared among our cultures of protozoa. Because of its large size it was thought that this organism might prove to be valuable bacteriological material for cytological study, since a review of the literature on this subject disclosed it to be in a considerable state of uncertainty. In addition, to our knowledge, ultracentrifugal force has not been applied to bacteria with the view of studying its effects upon the cytology of these organisms.

MATERIALS AND METHODS

Spirillum volutans appeared in a boiled wheat culture of mixed infusoria. The bacteria grew and multiplied rapidly when subcultured at intervals of about two weeks. Both normal and ultracentrifuged organisms were fixed in 100 per cent alcohol, Schaudinn's, Bouin's, Champy's and Regaud's solutions. They were stained in Heidenhain's hematoxylin, Delafield's hematoxylin, Regaud's hematoxylin, aceto-carmine, by Feulgen's method, in silver nitrate, in osmic acid and in nigrosin. In addition they were vitally stained by Janus green B, neutral red, methylene-blue and brilliant-cresyl-blue. They were also observed in the living unstained condition, in dark field and under polarized light; the micro-incineration technique was likewise carried out. The bacteria were centrifuged at 400,000 times gravity for 10 to 20 minutes in the air-driven ultracentrifuge developed by J. W. Beams at the University of Virginia, to whom we are indebted for having constructed the apparatus for us.

DESCRIPTION

Spirillum volutans is a large, quite rigid, corkscrew-shaped bacterium which is equipped with a group of flagella at each end (fig. 1). It moves in a rather tight spiral, the movement appearing to be quite without jerkiness. There are numerous granules and vacuole-like inclusions in the protoplasm which appear highly refractive or dark, depending upon the focus at which they are observed (fig. 4). Vital staining with nigrosin reveals these structures much more clearly than under ordinary unstained conditions (fig. 6).

Due to their rigid covering, these organisms do not break up nor are they greatly distorted by ultracentrifuging but all the granules and vacuoles are con-

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centrated in the centrifugal "troughs" of the body: the centripetal "crests" being nearly optically homogeneous (figs. 1, 2, 3, 5, and 7). In other forms, mitochondria (Beams and King, 1934), chromatin (Beams and King, 1938), plastids (Beams and King, 1935) and volutin (Patten and Beams, 1936) are heavier than the surrounding cytoplasm. This condition inhibits locomotion in *Spirillum* because of the unequal distribution of weight in the spirals; the organisms float "troughs" down, "crests" up and sway from side to side uncertainly. This reaction of the granules to high centrifugal forces indicates that they have a higher specific gravity than the surrounding cytoplasm. Furthermore, the internal cytoplasm cannot be of the same rigid architecture as the surface, since these experiments indicate that the viscosity of the internal cytoplasm is not unusually high as compared to that of other organisms. When removed from the ultracentrifuge and allowed to stand for 30 to 60 minutes the granules and vacuoles often completely redistribute.

The nature of the chromatin material in *Spirillum* is doubtful. Fixation in Schaudinn's fluid followed by Heidenhain's hematoxylin stain, demonstrates dark granules scattered throughout the organism as are also clear vacuole-like bodies (fig. 8). Similar granules are seen following the aceto-carmine technique (fig. 13). However, these bodies did not give a sufficiently satisfactory reaction with the Feulgen method to permit a definite decision as to whether they are questionably positive or completely negative. Undoubtedly they are what certain other investigators have interpreted as chromatin or chromidia, but still others have claimed that they are volutin (Lewis, 1940). From our studies we can only conclude that, in spite of their questionable reaction with the Feulgen method, they react to other chromatin stains (hematoxylin and aceto-carmine) more nearly like the chromatin of higher forms than any of the other granules demonstrated. On the other hand, hematoxylin is said to stain volutin (Taylor, 1937) and, as is the case with so many other staining reactions, it is not to be considered a specific stain for chromatin.

Following treatment of the living organism with Janus green B numerous small granules are also seen (fig. 10). These appear to be more numerous than the ones demonstrated by the hematoxylin and aceto-carmine methods. They do not appear to be volutin granules and they react to Janus green B in much the same way as do the mitochondria in cells of higher organisms. However, we hesitate to claim them to be mitochondria dogmatically on the basis of their reaction to Janus green B alone, since it is well known that this stain, despite the claims of some investigators, is not a specific stain for mitochondria. With Regaud's method (fig. 9) granules are demonstrated which appear much like those seen following Schaudinn's fixation and Heidenhain's hematoxylin staining.

Living organisms stained with methylene blue reveal granules which probably are volutin material (fig. 11). Organisms fixed in Schaudinn's fluid and subsequently stained in methylene blue (fig. 12) also show granules which appear to be somewhat larger than those stained vitally with methylene blue. However, the distribution of the two sets of granules is not unlike; so that they probably represent the same material, namely volutin (see Lewis, 1940).

A few darkly-colored bodies are found scattered in the cytoplasm following the osmic acid method used so extensively for demonstrating Golgi material in the cells of higher organisms (fig. 15). These appear to be vacuolar in nature and probably are not comparable to the Golgi material. They may be fat but in any case they probably are not comparable to the clear unstained vacuoles seen in the hematoxylin methods of Heidenhain and Regaud (figs. 8 and 9). Organisms incinerated according to the method of Scott (1937) reveal, under darkfield, masses of ash (fig. 14). Methods were not available to determine the chemical nature of the ash but it probably represents, as indicated by its distribution, the ash left by the volutin granules rather than any ash residue left by fats.

Spirillum volutans divides by transverse fission as may be seen in figures 2 and 3. In figure 2 apparently the "cell plate" has become sufficiently formed to prevent the passage of the centrifuged granules through it.

DISCUSSION

Good reviews of the nature of the chromatin material and the methods of division in bacteria are available and need not be repeated here (Guilliermond, 1907; Dobell, 1911; Lindegren, 1935; Stille, 1937; Knaysi, 1938; Lewis, 1940; and Beebe, 1941). Suffice to say that there are at present five views concerning the nature of the nuclear apparatus in bacteria as follows: (1) Nuclei similar to those demonstrated in higher plants and animals are absent. (2) The whole bacterial cell is regarded as homologous to the nucleus of the cells of higher organisms. (3) Bacteria possesses definite nuclei. (4) The chromatin of bacteria is scattered throughout the cell body of the organism in the form of small granules. (5) The bacterial nucleus may consist of naked rather than chromatin-encrusted gene strings.

For *Spirillum*, Bütschli (1902) has described a "central body" as the nucleus. This central body is considered as the endoplasm by most other authors. Swellengrebel (1909) holds that the chromatin of *Spirillum giganteum* is present either as scattered granules in the protoplasm or in the form of diagonal and zig zag bands; a similar view is held by Dimitroff (1926) for *Spirillum virginianum*. Dobell (1911) found scattered chromidia in *Spirillum monospora*, a filamentous nucleus in a spirillum from the gut of lizards and a spherical nucleus in a spirillum from the large gut of the cockroach. Lewis (1940) holds that chromatin is not demonstrable in *Spirillum volutans* but must be present in the form of a naked gene string.

At first view the evidence reported here seems to support the idea that the chromatin in *Spirillum* is in the form of scattered discrete granules. However, it must be kept in mind that, in our hands the Feulgen method did not give sufficiently clear information for us to state that a positive reaction was obtained with any of the structures in *Spirillum*. We are well aware of the fact that a positive reaction with chromatin bodies, previously described as such (Nakanishi, 1901), in other bacteria has been reported following the use of the Feulgen method (Stille, 1937; Piekarski, 1937). Bacteria which have been ingested by Para-

mecium (*Bacillus subtilis* ?) often show a positive Feulgen reaction similar to that reported by Stille (1937) for *B. subtilis*.

No evidences of any form of mitosis of the kind seen in higher organisms were observed by us. The method of division in *Spirillum* seems to be transverse binary fission resulting in the formation of two separate daughter cells. Neither division of the granules in the organism prior to cytokinesis nor the origin of new flagella could be observed. However, in keeping with the facts of division of higher organisms there might be expected an orderly division of the chromatin material if the principles of genetics operate in bacteria as they do in higher organisms. However, many species of protozoa, such as *Opalina*, show a condition in which the repeated division of the nuclear material precedes by considerable time the division of the cytoplasm. Such a state of affairs in which the widely dispersed chromatin material divides independently of cytoplasmic division might be easily imagined to occur in bacteria. However, it might also be argued that, in lower forms of life such as the bacteria, the chromatin or gene-carrying material is not differentiated at all, or that the chromatin is chemically different from that in higher forms, or that it is so diffuse that a positive staining reaction with the Feulgen method will not occur. This latter condition is known to be the case in the ova of certain invertebrates, where a negative Feulgen reaction has been reported at one stage yet chromatin which gives a positive reaction is known to be present at another stage. Cunda and Muniz (1929) found Feulgen-positive granules in *Bacillus anthracis* from a 16–18 hour old culture but none in the spores. Also Pokrowskaja (1931) found that *Pasteurella pestis* living parasitically gave only a diffuse nucleal reaction while under saprophytic conditions a positive reaction was obtained for a "true" nucleus which divided amitotically. This is somewhat similar to the conditions described by Schaudinn (1902) who held that in *Bacillus bütschlii* the chromatin was differentially scattered in the vegetative cell but present as a "true" nucleus in the spores. Beebe (1941) has described the nucleus as a compact Feulgen-positive mass in *Myxococcus xanthus*. This nucleus breaks up into chromosomes during division and undergoes autogamy before spore formation and meiosis during germination of the spores.

The gene string theory of Lindegren (1935; see also Lewis, 1940) is, indeed, of great theoretical interest. However, there is little, if any, cytological evidence to support such a view; in fact there is an abundance of evidence to show that demonstrable chromatin is present in certain bacteria; a condition which would, if true, make the above theory redundant for those forms at least. As regards the various views listed above concerning the nature of the chromatin of bacteria, in general, it is entirely possible, it seems to us, that the state of the chromatin may differ in different forms of bacteria. Therefore, if this should be the case, efforts to generalize too broadly on this question from the condition found in any individual group of bacteria would be folly.

Evidence for the presence of mitochondria in *Spirillum* as indicated by the methods used to demonstrate them in higher organisms is also questionable. As mentioned before, Janus green B stains small granules which may or may not be mitochondria. Efforts to demonstrate the Golgi apparatus in these organisms

using the osmic acid technique were in vain. Of course, this does not mean that it is not present, but only that it does not appear in the form characteristic of higher organisms. This is not surprising since in the protozoa and in plant cells in general, conflicting evidence for the presence or absence of Golgi material has been published.

In Heidenhain hematoxylin preparations, figures like those shown in plate 1 by Lewis (1940) are found. In addition to the darkly-staining granules many clear bodies are present which Lewis interprets as fat. The evidence presented in this paper cannot be easily brought to support this idea. The interpretation that these bodies are fat is based mainly on the fact that they stain with Sudan IV and Sudan black B. On the other hand our evidence, which indicates that they probably are not fat is based mainly on their reaction to centrifuging and their general failure to reduce osmic acid. If our observation that all of the granules and vacuoles move to the centrifugal pole is correct, they cannot be intracellular fat because fat being lighter than protoplasm generally would move to the centripetal pole. It could be, however, that they represent reserve food material around whose periphery fat might collect and in which case they might be heavier than the surrounding protoplasm. In osmic acid preparations only a few of these bodies are darkened among many unstained ones. The former cannot be the fat bodies referred to by Lewis because of their relatively small number. Our interpretation is that these osmified vacuoles may be fat, vacuoles of some other kind or possibly even artifacts.

To our knowledge evidence as to the effects of high centrifugal force upon the cytology of bacteria has never previously been reported. From the evidence presented here it is clear that the intracellular granules and vacuoles can be displaced and concentrated at the centrifugal regions of the cell and, upon removal of the organisms from the ultracentrifuge, are redistributed to an apparently normal condition. This is interpreted to mean that the granules are of different density from that of the cytoplasm which, compared to that of cells of other centrifuged organisms, does not seem to differ greatly in viscosity. It further demonstrates that normal locomotion of the bacteria can be impeded by the unequal concentration of the granules resulting in an asymmetrical distribution in weight in different portions of the body. We have subjected *Spirillum* to centrifugal forces of the order of 800,000 times gravity for 10 minutes without killing them. This indicates that molecular stratification in the organisms did not take place or that if such did occur, death was not the result. This is interesting in view of the fact that molecular displacement in non-living systems may occur readily in fields of such intense centrifugal force.

SUMMARY

1. All the granules visible in living *Spirillum volutans* are displaced to the centrifugal region upon ultracentrifuging. Therefore all these granules are heavier than the protoplasm surrounding them.

2. There are at least two different kinds of granules as is demonstrated by the fact that certain ones do not stain in iron hematoxylin while others do.

3. Certain of the large clear vacuole-like inclusions, which do not stain with iron hematoxylin, reduce osmic acid. However, this reaction does not take place in the great majority of these, so they cannot be regarded as fat. That they are heavier than the cytoplasm surrounding them is also opposed to their interpretation as fat. However, they may be reserve food with a peripheral layer of fat.

4. Although a clear differentiation cannot be made among the granules by staining in iron hematoxylin following fixation in Regaud's fluid, it is probable that some of the smaller ones, i.e. those staining vitally in Janus green B, may represent mitochondria or mitochondria-like bodies.

5. Chromatin responding positively to the Feulgen reaction has not been demonstrated in *Spirillum* nor has a definite identification of chromatin or nucleus been made. There is no reason for believing that the nucleus and chromatin must be present in uniform condition in all bacteria.

REFERENCES

BEAMS, H. W., AND KING, R. L. 1934 Effects of ultracentrifuging upon the mitochondria of the hepatic cells of the rat. Anat. Record, **59**, 395–401.

BEAMS, H. W., AND KING, R. L. 1935 The effect of ultracentrifuging on the cells of the root tip of the bean. Proc. Roy. Soc. (London) B., **118**, 264–276.

BEAMS, H. W., AND KING, R. L. 1938 An experimental study of mitosis in the somatic cells of wheat. Biol. Bull., **75**, 189–207.

BEAMS, H. W., AND KING, R. L. 1940 The air-driven ultracentrifuge, its application in biology. J. Roy. Microscop. Soc., Series III, **60**, 240–256.

BEEBE, J. M. 1941 The morphology and cytology of *Myxococcus xanthus*, N. sp. J. Bact., **42**, 193–223.

BÜTSCHLI, O. 1902 Bemerkungen über Cyanophyceen und Bakteriaceen. Arch. Protistenk., **1**, 41–58.

CUNDA, M., AND MUNIZ, J. 1929 Réaction nucléaire de Feulgen chez les Spirochétes et les Bactéries. Compt. rend. soc. biol., **100**, 951–954.

DIMITROFF, V. T. 1926 *Spirillum virginianum*. Nov. Spec. J. Bact., **12**, 19–49.

DOBELL, C. C. 1911 Contributions to the cytology of Bacteria. Quart. J. Microscop. Sci., **56**, 395–506.

GUILLIERMOND, A. 1907 La cytologie des bacteriens. Bull. inst. Pasteur., **5**, 273–283.

GUILLIERMOND, A. 1908 Contribution a l'étude cytologique des Bacilles endosporés. Arch. Protistenk., **12**, 9–43.

KNAYSI, G. 1938 Cytology of bacteria. Botan. Rev., **4**, 83–112.

LEWIS, I. M. 1940 The genus *Spirillum* Ehbg. with special reference to cell inclusions and the chromidial theory. J. Bact., **40**, 271–285.

LINDEGREN, C. C. 1935 Genetical studies of bacteria. I. The problem of the bacterial nucleus. Zentr. Bakt. Parasitenk., II, **92**, 40–47.

NAKANISHI, K. 1901 Ueber den Bau der Bakterien. Zentr. Bakt. Parasitenk., I, **30**, 97–110, 193–201, and 225–232.

PATTEN, R., AND BEAMS, H. W. 1936 Observations on the effect of the ultracentrifuge on some free living flagellates. Quart. J. Microscop. Sci., **78**, 615–635.

PIEKARSKI, G. 1937 Cytologische Untersuchungen an Paratyphus und Colibacterien. Arch. Mikrobiol., **8**, 428–439.

POKROWSKAJA, M. 1931 Zytologische Beobachtungen über den Dissoziationsprozess der Pestbazillen. Zentr. Bakt. Parasitenk., I., **119**, 353–361.

SCHAUDINN, F. 1902 Beiträge zur Kenntnis der Bacterien und verwandter Organismen. Arch. Protistenk., **1**, 306–343.

STILLE, B. 1937 Zytologische Untersuchungen an Bakterien mit Hilfe der Feulgenschen Nuclealreaction. Arch. Mikrobiol., **8**, 125–148

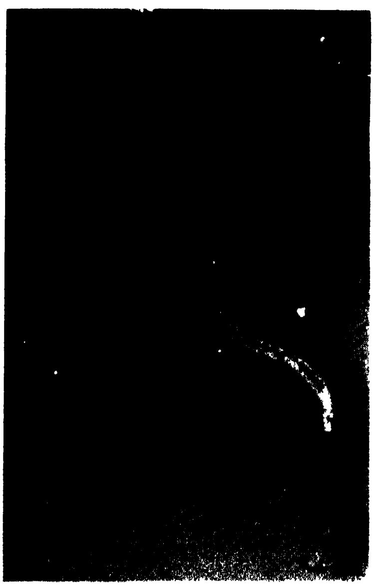
SCOTT, G. H. 1937 The microincineration method of demonstrating mineral elements in tissues. Microscopical Technique, Chap 11, Edited by C. E. McClung (Paul B. Hoeber, N. Y.).

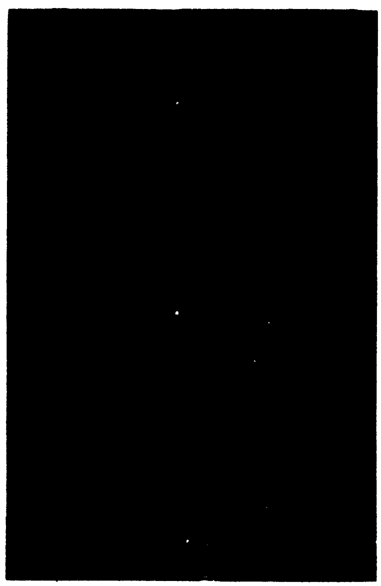
SWELLENGREBEL, N. H. 1909 Neuere Untersuchungen uber die vergleichende Cytologie der Spirillen und Spirochaten. Zentr. Bakt Parasitenk., I., **49**, 529–550.

TAYLOR, W. R. 1937 General botanical microtechnique. Chap. 4, Microscopical technique, edited by C. E. McClung (Paul B. Hoeber, N. Y.).

PLATE I

FIGS. 1–3. Photographs of centrifuged Spirillum, stained in Nigrosin and dried. The visible inclusions are concentrated in the centrifugal "troughs." In addition, figures 2 and 3 show stages in division.





(R. L. King and H. W. Beams: Ultracentrifugation and Cytology of S. Volutans)

PLATE II

FIG. 4. Normal living Spirillum. The inclusions show the usual random distribution.

FIG. 5. Centrifuged living Spirillum. The inclusions are packed into the centrifugal "troughs."

FIG. 6. Normal living Spirillum vitally stained in Nigrosin. Inclusions as in figure 4.

FIG. 7. Centrifuged living Spirillum vitally stained in Nigrosin. Inclusions as in figure 5.

FIG. 8. Spirillum fixed in Schaudinn's fluid, stained in iron hematoxylin.

FIG. 9. Spirillum fixed in Regaud's solution, stained in iron hematoxylin.

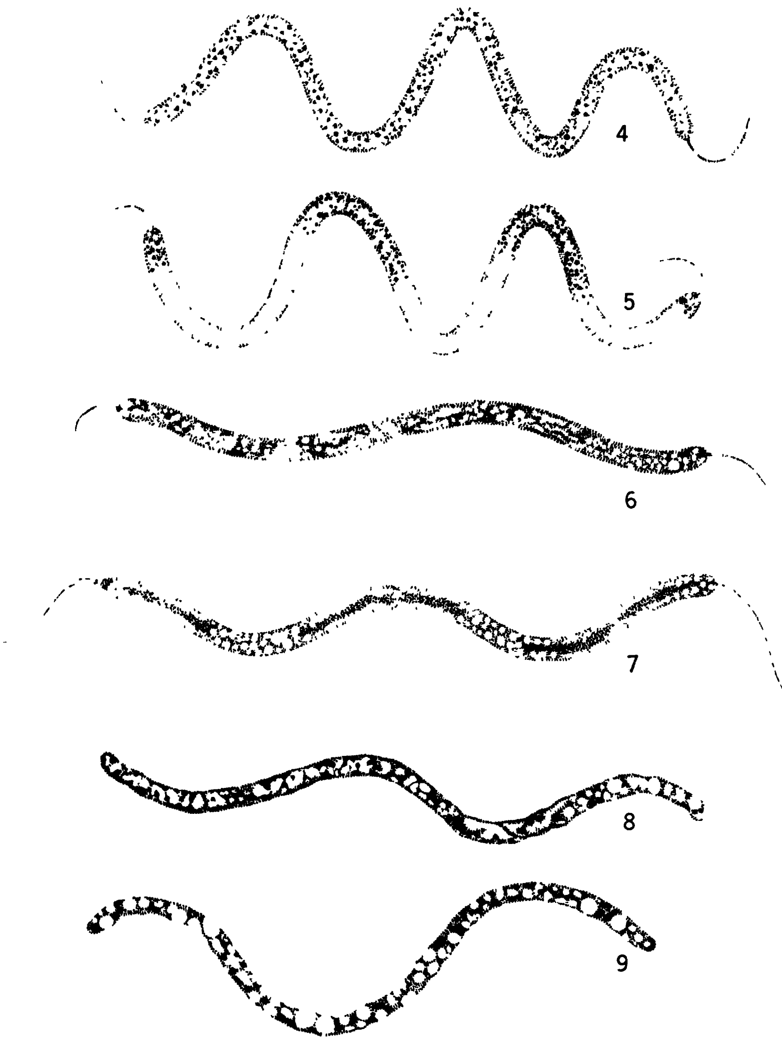


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PLATE III

FIG. 10. Normal living Spirillum, vitally stained in Janus green B.

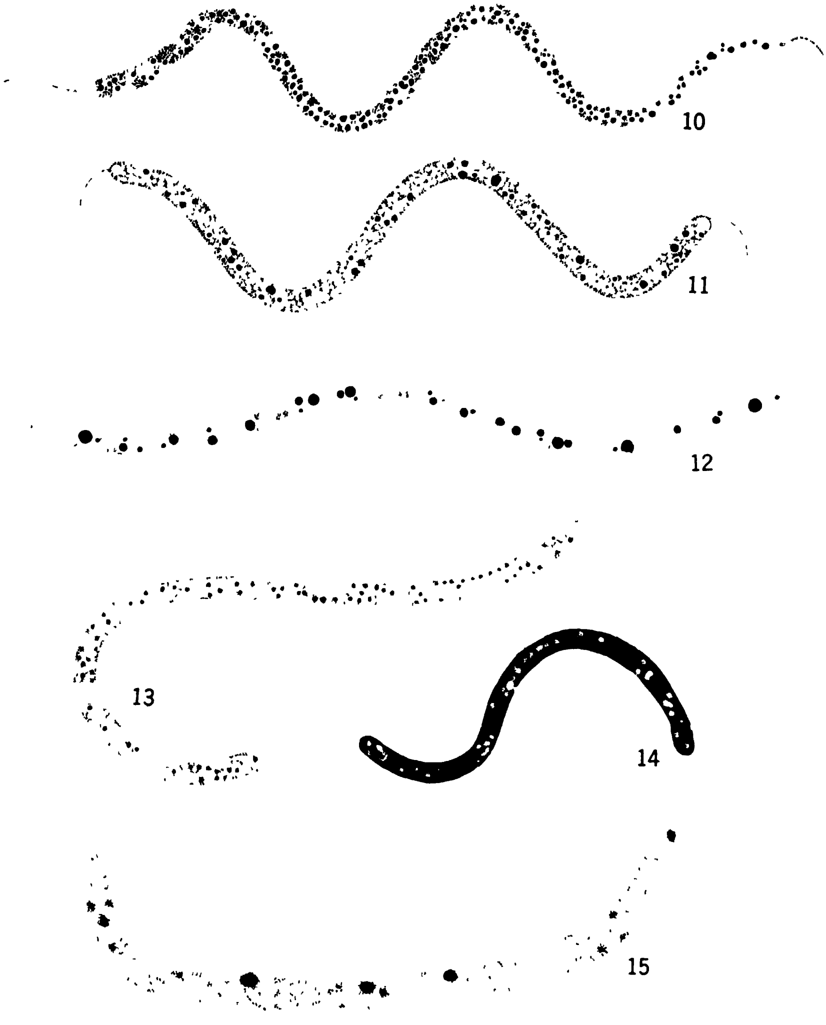
FIG. 11. Normal living Spirillum, vitally (?) stained in methylene blue.

FIG. 12. Spirillum fixed in Schaudinn's fluid and stained in methylene blue.

FIG. 13. Spirillum fixed and stained in aceto-carmine.

FIG. 14. Spirillum prepared by microincineration method. Ash in same relative position as larger inclusions. Drawn in dark field.

FIG. 15. Spirillum treated with osmic acid for seven days. Rarely a few inclusions are osmified.



(R. L. King and H. W. Beams: Ultracentrifugation and Cytology of S. Volutans)

THE STATUS OF BACILLUS SUBTILIS, INCLUDING A NOTE ON THE SEPARATION OF PRECIPITINOGENS FROM BACTERIAL SPORES

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At the Second International Congress for Microbiology the Nomenclature Committee agreed to the designation of *Bacillus subtilis*, Cohn, 1872, *emendavit* Prazmowski, 1880 as the type species of the genus *Bacillus* and recognized the Marburg strain as the type strain. The latter action was based on recommendations arising from Conn's (1930) study of the Michigan and Marburg strains. Soule (1932) previous to the meeting had presented the arguments for the adoption of the large-celled Michigan strain as the standard strain if the Committee were to accept *B. subtilis*, Cohn, 1872 as the type species of the genus. Since the Congress Lamanna (1940b) has reported that the Marburg strain was not to be distinguished from *Bacillus vulgatus*.

The present study was undertaken to determine whether it is probable that Cohn and Prazmowski observed a small-celled species and to learn if the deposited Marburg strain in the American Type Culture Collection is identical with *B. vulgatus*. The fifth edition of Bergey's Manual does not list *B. vulgatus* as a species. But the more recent work of Lamanna (1940b), Sievers and Zetterberg (1940), Sievers (1942), and data to be presented leave little doubt as to the eligibility of *B. vulgatus* to species rank.

ISOLATIONS FROM BOILED HAY INFUSIONS

Five samples of hay from four separate and distant barns were collected. Each sample was placed into a sterile two-liter flask and boiling water added until the hay was covered by a layer of water a few millimeters deep. The flasks were boiled for 15 minutes, cooled in tap water and incubated. A white fragile pellicle became visible in all cases at the end of 48 hours.

After two and four days of incubation a few drops of the hay infusions were transferred by sterile pipette to nutrient agar slants. Hanging drop preparations of growth from all the slants prepared from the 48-hour-old infusions showed only small motile cells.

Platings of the growths on the agar slants were made by the loop dilution technique. The resulting colonies were studied for differences. Two colonies of those that appeared to be representative of the types present in each sample of hay at the end of 48 and 96 hours were picked onto nutrient agar. An incubation temperature of 25°C. was used in the entire described procedure.

The isolated pure cultures were subjected to a morphological, physiological and serological (spore as antigen) study. The methods of study and criteria for des-

ignating species have been outlined elsewhere (Lamanna, 1940 a, b, c). Table 1 lists the results. It is evident that in a boiled hay infusion one will find small-celled types of aerobic spore-formers within the first 48 hours of incubation with large-celled types appearing later. The conclusion of Conn (1930) that Cohn and Prazmowski observed a small-celled strain in boiled hay infusion is tenable and the Nomenclature Committee's action in recognizing a small-celled type as the true *B. subtilis* is justified.

However, it is also evident that depending on a great number of variables one or another small-celled species may predominate in a boiled hay infusion within 48 hours, and not always as a pure culture. It is, therefore, impossible to be certain as to exactly which small-celled species Cohn and Prazmowski called *B. subtilis*.

TABLE 1

Isolation of aerobic spore-formers from hay infusion boiled 15 minutes and incubated at 25°C

WEIGHT OF HAY SAMPLE	1	2	3	4	5
	507 g.	467 g.	609 g.	510 g.	670 g.
Species isolated at end of 48 hours	*B. vulgatus*† *B. vulgatus**	*B. agri*† *B. agri*	*B. vulgatus* *B. agri*	*B. vulgatus* *B. vulgatus*	*B. vulgatus* *B. vulgatus*
Species isolated at end of 96 hours	*B. vulgatus* *B. vulgatus*	*B. cereus*‡ *B. cereus*	*B. vulgatus* *B. vulgatus*	*B. cereus*-like‡ *B. cereus*-like	*B. cereus*-like *B. cereus*-like

* Atypical, does not reduce nitrates; starch hydrolysis negative.
† Small-celled.
‡ Large-celled.

Prazmowski observed the method of spore germination of his organism which is helpful in delimiting his species. But as one characteristic not correlated with others it is only helpful rather than conclusive evidence. Thus, in choosing the type strain to represent the species *B. subtilis* one is not guided by knowledge of all the necessary facts. The conclusion reached is never unchallengeable and must rest in part on opinion and convenience. In the material that follows it will be demonstrated that the Marburg strain accepted as the type is actually *B. vulgatus*, and that it would be most convenient and do no violence to the facts on hand if a strain labeled as *B. subtilis* by Lawrence and Ford (1916) is accepted as the more likely representative of the organisms studied by Cohn and Prazmowski.

The following strains are reported on:

S 8 *B. subtilis*, Ford. Received by Soule from Ford.

C 4 *B. subtilis*, Marburg strain, from H. J. Conn.

St *B. vulgatus* from Washington State College Collection.
C 5 *B. vulgatus* from H. J. Conn.
UA3, UA7, UA11 probably *B. subtilis* from L. B. Schweiger.

Purchased from the American Type Culture Collection:

102 *B. subtilis*, W. W. Ford, through American Museum of Natural History. Sold to private collection. Reobtained by A T C C and sent to present author.

465 *B. subtilis*, N. M. Harris, Ottawa.

6051 *B. subtilis*, H. J. Conn. The Marburg strain, the type strain of the species.

6633 *B. subtilis*, N. R. Smith. Bureau Plant Industry, U.S.D.A. strain no. 231.

6598 *B. subtilis*, Veterinary Corps, Army Medical School, Washington, D. C. From blood stream of a horse.

4529 *B. vulgatus* Trevisan. From W. W. Ford. From milk 1916.

6984 *B. vulgatus* Trevisan, var. *hydrolyticus* Hermann. From Hermann, German University of Prague.

From Dr. Ruth Gordon the following strains were received:

231 *B. subtilis*, Smith 104, from Kellerman, 1912.

238 *B. vulgatus*, Smith 164, American Type Culture Collection 123, A. M. N. H. 725, from Ford.

Table 2 records the data obtained. The strains are easily separable into two distinct groups. The characterization of each group is based on a number of criteria which are probably of sufficient significance to warrant labeling each group as a natural species. In a personal communication Prof. Knaysi (1941) who has studied the Marburg and Ford strain (S 8) of *B. subtilis* has stated that in his opinion the two cannot be confused. Schweiger (1942) has written me that he has found no serological relationship between vegetative cells of strain UA11, and strains UA3, UA7.

It should be noted that strains of *B. vulgatus* have been named by persons isolating them at times as *vulgatus*, and sometimes as *subtilis*, whereas those strains resembling Ford's *subtilis* have been named *B. subtilis* and never *vulgatus*.

The reasons for this may be explicable. The original separation of *B. vulgatus* (Flügge, 1886; 5 ed. Bergey's Manual, 647–649) was on the basis of pigment formation, especially the deep red color developed on potato slants. However, the pigment formation is not a constant characteristic (Lehmann and Neumann, 1920). Gordon and Smith (1942) have reported loss of pigmentation by *Bacillus aterrimus* which species Lamanna (1940b) had pointed out resembled the Marburg strain and *B. vulgatus*. Wechsler (1939) has shown that pigmentation of strains of *B. subtilis* and *B. vulgatus* in both cases is related to tryosine metabolism, but that *vulgatus* is the more vigorous pigment former. Console and Rahn (1938) noted that their usually non-pigmented culture of the Marburg strain often dissociated into a pigment former. We may conclude that when a strain of *B. vulgatus* upon isolation has formed abundant pigment it was labeled *B.*

vulgatus or as a related variety but when pigmentation was absent or meagre it was labeled *B. subtilis* or Marburg strain. The difficulty to date has been that insufficient criteria existed for proper classification.

The question that remains is whether the Marburg strain should be recognized as the type strain of *subtilis*. If it is, then *B. vulgatus* must be discarded and a

TABLE 2

STRAIN	NAME GIVEN BY AUTHOR WHO MADE ISOLATION	FERMENTATION OF: Glucose	Sucrose	Lactose	STARCH HYDROLYSIS	MODE OF SPORE GERMINATION	GROWTH AT °C.: 55	15	VOGES-PROSKAUER REACTION*	SEROLOGICAL TESTS ANTSIERA PREPARED AGAINST SPORES OF STRAIN: Agglutination: S8	C4	St	*B. agri*	Precipitation: S8	C4	St	*NB. agri*	NITRATE REDUCTION TO NITRITES
Identified as strains of Ford and Lawrence's *B. subtilis*																		
S8	*subtilis*	+	+	0	+	Equatorial without splitting along the transverse axis	+	0	+	+	0	0	0	+	0	0	0	+
6598	*subtilis*	+	+	0	+		+	0	+	+	0	0	0	+	0	0	0	+
UA3	*subtilis*	+	+	0	+		+	0	+	+	0	0	0	+	0	0	0	+
UA7	*subtilis*	+	+	0	+		+	0	+	+	0	0	0	+	0	0	0	+
Identified as strains of *B. vulgatus*																		
102	*subtilis*	+	+	0	+	Equatorial with and without splitting along transverse axis (ratio 1.5 to 1)	+	+	+	0	+	+	0	0	+	+	0	0
6051	Marburg strain	+	+	0	+	Equatorial with splitting along the trans verse axis	+	+	+	0	+	+	0	0	+	+	0	+
C4	Marburg strain	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+
C5	*vulgatus*	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+
St	*vulgatus*	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+
6084	*vulgatus*	+	+	0	+		v.sl. ±	+	+	0	+	+	0	0	+	+	0	+
4529	*vulgatus*	+	0	0	+		+	+	0	0	+	+	0	0	n.r.	+	0	+
238	*vulgatus*	+	+	0	+		0	0	+	0	+	+	0	0	+	+	0	+
231	*subtilis*	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+
465	*subtilis*	+	+	0	+		0	+	v.sl.+	0	+	+	0	0	+	+	0	+
6633	*subtilis*	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+
UA11	*subtilis*	+	+	0	+		0	+	+	0	+	+	0	0	+	+	0	+

* Barritt's method.

+, positive; 0, negative; v.sl., very slight; n.r., not run; ±, repeated tests gave variable results.

new name invented for Ford's *B. subtilis*. It is recommended that *B. vulgatus* be retained as a species and Ford's *subtilis* taken as the type of small-celled spore-former observed by Prazmowski. Prazmowski's and Brefeld's figures of spore germination of *subtilis* (reprinted in Soule, 1932) can be interpreted as representing spore germination by equatorial germination without splitting along the transverse axis. Ford's strain germinates in this fashion. The Marburg strain

and *B. vulgatus* germinate equatorially with splitting along the transverse axis (Lamanna, 1940a). Thus, even though Prazmowski did not give an adequate description of his organism by present day standards, it can be recognized that Ford's strain resembles it more closely than the Marburg strain.

Previous to the Congress R. E. Buchanan (1935) in presenting his proposal that a committee be appointed to fix upon the standard strain of *B. subtilis* stated that the committee should,

> "(I) Secure, if such is in existence, a culture derived from the original culture isolated by Cohn, and which agrees with his description.
>
> (II) If (I) is not possible, secure a culture which has been described and accepted as *Bacillus subtilis*, and which agrees as closely as possible with the original description by Cohn."

No subcultures of Cohn's original isolation exist. Acceptance of the Ford strain of *subtilis* would coincide with the spirit of the above remarks. The Ford strain is available, has been described and accepted by many as *B. subtilis*, agrees as closely as the Marburg strain to Cohn's original description, and more closely with Prazmowski's and Brefeld's figures of spore germination.

Strains S8 and 102 were named *B. subtilis* by Ford. Yet the two are different (see table 2). The author prefers to choose S8 as representing Ford's *subtilis* because it came to him through only one other person (Soule). Strain 102 went from Ford to the American Museum of Natural History, then to the A.T.C.C., sold to a private collection, back to A.T.C.C. and on to the present author. In addition, strain S8 in cell size is smaller than cells of similar age of 102. Consequently S8 agrees more closely with Ford and Lawrence's description of *B. subtilis*.

Strain 102 unlike other *B. vulgatus* strains does not reduce nitrates and shows equatorial spore germination, both with and without splitting along the transverse axis. But in the other aspects of its natural history it resembles the *B. vulgatus* more closely than any other species and so is placed with them. The problem of intermediate types is a difficult one in bacteriology and the aerobic spore-formers apparently present no exception in this regard. A rational system of classification will base separation on a study of a wide variety of properties and place intermediates with the groups they resemble most closely rather than lead to the creation of innumerable species or sub-species.

PRECIPITINOGENS FROM BACTERIAL SPORES

The agglutination tests reported in table 2 employed bacterial spores mixed with rabbit antisera prepared against spores of the listed strains. The method of running the agglutination test has been reported (Lamanna, 1940b). The precipitation test was set up by layering varying dilutions of antigen solution in a set of tubes (5 by 50 mm.) containing antiserum diluted with one part of physiological salt solution. Appropriate saline and normal serum controls were included. The appearance of a ring of flocculus within one hour of incubation at 37°C. or during subsequent overnight storage in a refrigerator was read as a positive test.

A negative result in table 2 means that no dilution of antigen gave an observable precipitate.

As antigen for the precipitation test 10 ml. of a physiological saline suspension of spores of turbidity equivalent to one ml. of a one-percent barium chloride solution added to nine ml. of one-percent sulphuric acid was pipetted into a pyrex test tube. The spores were centrifuged out, resuspended in five ml. of N/20 hydrochloric acid containing 0.85 percent sodium chloride, and boiled for 30 minutes. The spores were then centrifuged down and the supernatant neutralized with N/1 sodium hydroxide. After centrifuging the supernatant to remove a precipitate, which at times appeared upon neutralization, the resulting clear liquid was used, diluted and undiluted, as antigen for the precipitation test. The procedure is essentially similar to the Lancefield (1928) method for separating soluble C substance from hemolytic streptococci.

The precipitinogen present in the clear liquid can also be separated from the cell by boiling the spores in 1/10N hydrochloric acid. Normal acid gives no precipitinogen and is presumed to destroy it. Spores boiled for 30 minutes in the N/20 acid stained by Dorner's method appear no different from untreated homologous spores.

The antigen gives positive Molisch, Biuret, and xanthoproteic tests. One may postulate that the antigen is a carbohydrate carried as a prosthetic group in a protein molecule. Studies are underway to define the nature of the spore antigen more precisely and we hope to publish in the future results of studies on the presence or absence of the antigen in vegetative cells. At that time it will be proper to discuss the biological significance and taxonomic utility of these isolated spore precipitinogens.

CONCLUSIONS

During the first 48 hours of incubation (25°C.) of a boiled hay infusion small-celled types of aerobic spore-formers predominate.

The Marburg strain of *Bacillus subtilis* is identical with *Bacillus vulgatus*. *B. subtilis* of Ford and Lawrence (1916) is distinct from *B. vulgatus*. Both deserve rank as species.

It is recommended that Ford and Lawrence's organism be recognized as the standard strain of *B. subtilis*, Cohn, *emendavit* Prazmowski.

By boiling in N/10 or N/20 hydrochloric acid it is possible to separate precipitinogens from spores of *B. subtilis* and *B. vulgatus*. The antigen of each species does not cross precipitate with spore antiserum of the heterologous species.

BIBLIOGRAPHY

Barritt, M. N. 1936 The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. J. Path. Bact., **42**: 441–454.

Bergey's Manual of Determinative Bacteriology. 1939 5 ed., Williams & Wilkins Co.

Breed, R. S., and St. John-Brooks, R. 1935 Report on proposals submitted by R. E. Buchanan and H. J. Conn relative to the conservation of *Bacillus* as a bacterial generic name, fixing of the type species, and of the type or standard culture. Zentr. Bakt. Parasitenk., II, **92**: 481–490.

Cohn, F. 1872 Beiträge zur Biologie der Pflanzen, **1**: 175.

Cohn, H. J. 1930 The identity of *Bacillus subtilis*. J. Infectious Diseases, **46**: 341–350.

Consolе, A. D., and Rahn, O. 1938 A study of gelatin digestion by *Bacillus subtilis*. J. Bact., **36**: 47–52.

Flügge, C. 1886 Die Mikroorganismen. Leipsig.

Gordon, Ruth E., and Smith, N. R. 1942 The relation of *Bacillus aterrimus* and *Bacillus niger* to *Bacillus subtilis*. J. Bact., **43**: 55.

Knaysi, Geo. 1941 Personal communication.

Lamanna, C. 1940a The taxonomy of the genus *Bacillus*. I. Modes of spore germination. J. Bact., **40**: 346–359. 1940b II. Differentiation of small-celled species by means of spore antigens. J. Infectious Diseases, **67**: 193–204. 1940c III. Differentiation of the large-celled species by means of spore antigens. Ibid., **67**: 205–212.

Lancefield, Rebecca C. 1928 The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type specific substance in extracts of *Streptococcus haemolyticus*. J. Exptl. Med., **47**: 91–103.

Laubach, C. A., Rice, J. L., and Ford, W. W. 1916 Studies on aerobic spore bearing non-pathogenic bacteria. Part II. J. Bact., **1**, 493–534.

Lawrence, J. S., and Ford, W. W. 1916 Studies on aerobic spore bearing non-pathogenic bacteria. Part I. J. Bact., **1**: 273–320.

Lehmann, K. B., and Neumann, R. O. 1920 Atlas and Grundriss der Bakteriologie 6 ed., München.

Schweiger, L. B. 1942 Personal communication.

Schweiger, L. B., Trainer, D., and Evelith, D. F. 1941 A descriptive study of a spore-forming bacillus isolated from actinobacillotic lesions. J. Bact., **42**: 285–286.

Second International Congress for Microbiology, London, 1936. Report of Proceedings. Reprinted in J. Bact., **33**: 445–447, 1937.

Sievers, Olof. 1942 Investigations into the antigenic characters of *B. mesentericus* and *B. subtilis*. J. Bact., **43**: 305–312.

Sievers, O., and Zetterberg, Bo. 1940 A preliminary investigation into the antigenic characters of spore-forming aerobic bacteria. J. Bact., **40**: 45–56.

Soule, M. H. 1932 Identity of *Bacillus subtilis*. J. Infectious Diseases, **51**: 191–215.

Wechsler, Leona. 1939 Factors affecting the formation of melanin from tyrosine by some members of the genus *Bacillus*. Master's thesis, Cornell University; Personal communication.

NOTES

ANAEROBIC PLATES

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Several methods of preparing plate cultures of anaerobes without using sealed jars have been described. Fortner's method is well known. Krumwiede poured agar into the top of a Petri dish instead of the bottom and sealed it with the flat side of the bottom next to the agar. Diffusion of oxygen from the edges, the control of contaminants on the exposed agar, the difficulty of using the plate when opened, and the lack of surface colonies are objections. Brewer devised a special glass top for Petri dishes which left an air space and provided a partial seal by imbedding a glass flange in agar. The medium used, a modification of his excellent thioglycollate medium, took care of residual oxygen and seepage. The tops are heavy and are easily broken, they must be especially purchased, the pocket of air is irregular even with an exact quantity of agar, and the medium must be one which makes the residual oxygen ineffective.

The method herein described consists of the preparation of a streaked or poured plate culture of anaerobes or suspected anaerobes by the same methods used for aerobes. Any medium on which anaerobes will grow, if oxygen is removed, is suitable.

Anaerobic conditions are provided continuously with a small Petri dish, 75 mm. in diameter and 10 mm. high, in which *Serratia marcescens* is streaked on ordinary agar. The edge of the inoculated half of the small plate is pressed with a slight turning motion into the agar of the larger plate, trapping air between the two surfaces of agar and sealing, except for diffusion of oxygen through a layer of agar, under the edge, and up through another layer of agar. It takes but a short time for *Serratia marcescens* to consume the residual oxygen and any seepage is consumed immediately.

After incubation, the small plate is removed carefully, rotating first to loosen so that a circle of agar will not stick to the small plate. A bit of Scotch tape on the small plate will permit its removal without risk of touching the inoculated surface. Thereafter the anaerobic conditions are lost but the grown culture on agar may be handled for isolation and examinations at the time preferred for study with the same ease and the same technic that is used for aerobic cultures on plates.

This method for the cultivation of anaerobes has been tried with medical, dental, and pharmacy students none of whom had cultivated anaerobes before. It requires a minimum of experience and equipment and is convenient.

SEROLOGICAL RELATIONSHIPS BETWEEN DIPLOCOCCUS PNEUMONIAE AND HEMOPHILUS INFLUENZAE

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The knowledge of the serological relationships of various pneumococcal types to other species is continuing to increase. Reported observations of such relationships are listed in table 1.

In April 1942 an *Hemophilus influenzae* type a was isolated from the cerebrospinal fluid and nasal secretion collected from a child hospitalized because of a fractured skull. This organism exhibited a definite "Quellung" reaction when mixed with rabbit antipneumococcus type 6 serum. This finding was confirmed by Margaret Pittman who obtained positive results with two out of eight lots of rabbit antipneumococcus type 6 sera. Further studies have demonstrated that the inability of certain lots of serum to produce the reaction is due to the

TABLE 1

OBSERVERS	YEAR	PNEUMOCOCCUS TYPE	SEROLOGICALLY RELATED ORGANISM
Avery, Heidelberger and Goebel	1925	2	Friedländer's bacillus type B.
Sugg and Neill	1929	2	*Saccharomyces cerevisiae*
Dingle	1934	2	*Bacterium leptisepticum*
Barnes and Wight	1935	1	*Escherichia coli* (mucoid)
Sugg and Hehre	1942	2, 20 and 12	Leuconostoc mesenteroides
Kauffmann and Langvad-Nielson	1942	35, 35A and 35B	Salmonella, type kirkee

absence of immune substance for pneumococcus type 6b. It has been demonstrated that the cross reaction is reciprocal between pneumococcus type 6b and *H. influenzae* type a. The present evidence is that pneumococcus type 6b contains at least two carbohydrate components one in common with pneumococcus type 6a and the other with *H. influenzae* type a. It has been impossible to obtain a cross reaction in either direction between pneumococcus type 6a and *H. influenzae* type a.

In July 1942 an *H. influenzae* type c was isolated from the sputum of a patient hospitalized because of a respiratory infection. This organism when mixed with rabbit antipneumococcus type 21 serum exhibited a definite "Quellung" reaction. This reaction was obtained with two different lots of serum. This finding was confirmed by Pittman using two additional lots of serum. The present evidence does not warrant a statement concerning the reciprocity of the reaction between *H. influenzae* type c and pneumococcus type 21.

Conclusion:

An interesting serological relationship between certain pneumococci and *H. influenzae* has been demonstrated. The present evidence is that there is a

common carbohydrate component shared by pneumococcus type 6b and *H. influenzae* type a. The serological relationship between *H. influenzae* type c and pneumococcus type 21 has not, as yet, been proven to be reciprocal. These findings gain added significance when it is realized that in routine pneumococcal typing procedures as performed in diagnostic laboratories the morphological similarity of pneumococci and *H. influenzae* may be confusing. Investigations involving reciprocal absorption tests with the respective systems are in progress and the results obtained will be published in more detail.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN MISSOURI BRANCH

Bevo Building, Anheuser-Busch, Inc., St. Louis, Mo., April 14, 1942

Genetics of Yeast. *C. C. Lindegren*, Anheuser-Busch, Inc., St. Louis, Mo.

A yeast hybrid was made by mating a very stable and a relatively stable form of *Saccharomyces*. The hybrid proved to be exceptionally unstable, producing mutants in great abundance, with the general characteristics of the two parents as well as a large number of inferior types.

Exhibit and Discussion of Yeast Derivatives. *K. L. Cartwright*, Anheuser-Busch, Inc., St. Louis, Mo.

Yeast can be used to produce protein from ammonia and to convert sucrose to glycogen, yeast gum, alcohol, glycerol, acids, fats, and sterols. Yeast ash is composed chiefly of potassium, phosphorus, magnesium, and calcium.

Synthesis and storage of the vitamins of the B-complex and ergosterol by yeast, and the differences between different species were discussed. The use of yeast to produce medicinals such as, l-ephedrine, histamine, glutathione, lecithin, and purine-pyrimidine bases is reviewed.

The Effectiveness of Commercial Ultraviolet Ray Sterilizing Cabinets. *L. C. Tobin*, Anheuser-Busch, Inc., St. Louis, Mo.

Various studies were conducted on three commercial ultraviolet ray sterilizing cabinets. A 24-hour broth culture of *Escherichia coli* and a 96-hour culture of *Bacillus subtilis* were used as test organisms. Naturally and artificially contaminated glasses exposed for varying lengths of time, showed consistent reduction in number of bacteria, although sterile glasses were not always produced.

Suspensions of the test organisms spread on sterile slides and exposed to the ultraviolet rays showed approximately the same degree of reduction as did the glasses. Effectiveness of the exposure varied with the initial number of organisms, and with the type of cabinet used.

Bacteriological Control at Anheuser-Busch, Inc. *John B. Rehm*, Anheuser-Busch, Inc., St. Louis, Mo.

The bacteriological problems encountered in production of various products and the methods used in solving these problems were discussed. Corn syrups, starches, beer, pharmaceutical yeasts and yeast concentrates, malt syrups, and other products were briefly considered. Air examination by means of the Wells Air Centrifuge and microbiological procedures for assay of vitamin products received special attention.

The Commercial Production of Baker's Yeast. *D. F. Sager*, Anheuser-Busch, Inc., St. Louis, Mo.

The manufacture of Baker's Yeast was traced from its beginning as a laboratory culture through the various steps of commercial production. A motion picture depicting the process accompanied the discussion.

STUDIES OF BACTERIA FROM FERMENTING EGG WHITE AND THE PRODUCTION OF PURE CULTURE FERMENTATIONS[1]

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In a previous study (1942) an investigation was made of the bacterial and chemical changes taking place during the natural fermentation of egg white, a preliminary step in the commercial processes for preparing dried egg albumen. This study established rather conclusively that predominance of bacteria of either the *Aerobacter* or *Escherichia* genus is necessary from the standpoint of the production of a high quality dried albumen from naturally fermented egg white. Other bacterial types were occasionally encountered in experimental laboratory fermentations but it was found that when species of *Proteus* or *Pseudomonas* were present in large numbers during such fermentations, a dried albumen of inferior quality was usually obtained.

The cultures isolated in this investigation have been studied more thoroughly with the object of confirming the tentative classifications originally made, and determining their effects on sterile egg white in pure culture.

The results of microscopic and cultural studies on twenty strains tentatively classified in the genus *Aerobacter* are given in Table 1.

From the data given in this table it appears that 12 of these cultures are strains of the species *Aerobacter aerogenes* (Bergey, 1939). They produce acid and gas from glycerol and do not liquefy gelatin. Seven strains were motile and five were non-motile. Eight strains were encapsulated. They all fermented glucose, mannose, and galactose, the three sugars known to be present in egg white. They also fermented sucrose, maltose, arabinose, raffinose, cellobiose, mannitol and starch, although six strains failed to ferment dulcitol. They all brought about acid coagulation in litmus milk and produced nitrites from nitrates. Only one strain produced indole in tryptone broth.

The remaining eight strains may also be *Aerobacter aerogenes* since they are not exactly typical of the other species in this genus, namely, *Aerobacter cloacae* (Bergey, 1939). They do not liquefy gelatin. Neither do they ferment glycerol, starch or dulcitol. They are all actively motile by virtue of peritrichous flagella and none have capsules. It is believed, therefore, that they resemble *Aerobacter cloacae* more closely than *Aerobacter aerogenes* and should be classified as strains of the former. All eight ferment glucose, mannose and galactose, with the production of acid and gas, fully as vigorously as the 12 typical strains of *Aerobacter aerogenes*.

The results of similar studies with 13 isolations tentatively classified as belonging to the genus *Escherichia* are given in table 2.

[1] Agricultural Chemical Research Division Contribution no. 63.

The data recorded in table 2 show that all 13 strains of this group utilized citric acid as a sole source of carbon. Only one produced gas in Eijkman's broth at 45°C. This particular culture did not produce indole in tryptone broth. Seven of the 13 strains did produce indole and nine of them produced acetyl-methyl-carbinol. The production of acetyl-methyl-carbinol is not commonly encountered with methyl-red positive cultures but has been reported by Parr (1938) for strains of *Escherichia fruendii*. Three of the 13 strains had well-developed capsules. All were motile. There would seem to be little doubt that all of the 13 cultures should be classified as strains of *Escherichia freundii*. It should be noted that all strains fermented glucose, mannose and galactose.

One surprising feature of these studies was the absence of true fecal types of the *Escherichia* genus in this group. These investigations were not extensive enought to say at this time that these species are consistently absent in naturally fermented egg white; it is possible that under conditions resulting in this fermen-

TABLE 1

*Aerobacter group, colonies on eosin-methylene-blue agar consistently of the Aerobacter type, non-spore-forming, gram-negative, short rods**

SPECIES	FLAGELLA STAIN GRAY'S	MOTILITY HANGING DROP	CAPSULE STAIN ANTHONY'S	GLYCEROL BROTH	STARCH BROTH	DULCITOL BROTH	INDOLE TEST
A. aerogenes (12 cultures)	Not made	7+ 5−	8+ 4−	+	+	6+ 6−	1+ 11−
A. cloacae (8 cultures)	+	+	−	−	−	−	−

* All cultures fermented lactose with the production of acid and gas, utilized citric acid as the sole source of carbon; were methyl-red negative, Voges-Proskauer positive, reduced nitrates, produced an acid curd in litmus milk and a yellowish growth on potato slants. They fermented with the production of acid and gas, glucose, mannose, galactose, maltose, sucrose, cellobiose, raffinose, arabinose and mannitol broths.

tation they are completely overgrown by the strains of *Citrobacter* (*Escherichia freundii*) and *Aerobacter*. This possibility is being investigated.

Five cultures were isolated that were tentatively classified as belonging to the genus *Proteus*. Further studies on these organisms have failed to reveal clearly the exact species with which they should be identified. However, the results obtained tend to confirm the initial presumption that they resemble the genus *Proteus* more closely than any other genus now recognized. The results of these studies are given in table 3.

The data shown for the five strains would indicate that there may exist a heretofore undescribed group of organisms intermediary between *Aerobacter* and *Proteus*, for all five cultures have characteristics in common with both genera. In appearance, their agar colonies and their growths on agar slants resemble *Aerobacter cloacae*. They produce acetyl-methyl-carbinol, a characteristic more commonly associated with *Aerobacter* than *Proteus*. This property has been assigned to *Proteus bombycis*, a species listed in the appendix, for the genus *Pro-*

teus, in Bergey's Manual (1939), but, unlike *Proteus bombycis*, these cultures do not have well-defined capsules. All five of the strains fermented mannitol, although two of them did not produce gas. Since they also ferment sucrose it may be that they should be identified as strains of *Proteus hydrophilus* or *Proteus ichthyosmius*; however, they did not produce indole in tryptone broth, or fishy odors in milk, and did not grow on potato slants according to the descriptions

TABLE 2

*Escherichia group, colonies on eosin-methylene-blue agar originally coli-like, non-spore forming, gram-negative, short rods**

SPECIES	CAPSULE STAIN ANTHONY'S	EIJKMANS BROTH AT 45°C.	VOGES-PROSKAUER TEST	INDOLE TEST	DULCITOL BROTH	STARCH BROTH
E. freundii (13 cultures)	3-encapsulated		8+			
		12−	1 weakly +	7+	5+	5+
	10-no capsules found	1+	4−	6−	8−	8−

* All cultures appeared to be actively motile in hanging drop, fermented lactose producing acid and gas, utilized citric acid as a sole source of carbon, reduced nitrates and were methyl-red test positive. None liquefied gelatin. They all produced an acid curd in litmus milk and abundant yellow growths on potato slants. All produced acid and gas in glucose, mannose, galactose, sucrose, maltose, cellobiose, raffinose, arabinose, mannitol and glycerol broths.

TABLE 3

*Proteus group, pleomorphic, actively motile, gram-negatiue rods, fermenting glucose and sucrose but not lactose**

SPECIES	GELATIN STAB.	VOGES-PROSKAUER	METHYL RED TEST	MANNITOL BROTH	GALACTOSE BROTH	MANNOSE BROTH	ARABINOSE BROTH	RAFFINOSE BROTH	GLYCEROL BROH	SALICIN BROTH
Proteus sp. (5 cultures)	3-infundibuliform and 2-stratiform liquefaction	3+ 2 weakly +	3− 2+	3+ with acid and gas 2+ with acid but no gas	3+ with acid and gas 2+ with acid but no gas	3+ with acid and gas 2+ with acid but no gas	2− 2+ with acid & gas 1+ with acid but no gas	2− 3+ with acid and gas	1+ with acid and gas 4+ with acid but no gas	2+ with acid & gas 3+ with acid but no gas

* All cultures showed peritrichous flagella with Gray's stain and produced pearl white spreading growths on agar plates and slants and creamy white spreading growths on potato slants. They all decomposed urea, reduced nitrates, and were indole negative. None showed capsules with Anthony's stain or fermented dulcitol broth. All decolorized and peptonized litmus milk and fermented maltose broth with the production of acid and gas.

given for these species. Two of the cultures did not ferment arabinose, raffinose, or dulcitol. The other three fermented arabinose and raffinose but not dulcitol. All five fermented glucose, mannose and galactose.

Two strains of *Serratia* were isolated. These possessed all of the characteristics assigned to *Serratia marcescens*. The results of the studies on these two isolations, along with data compiled on a number of strains of *Pseudomonas* of

the fluorescent type isolated also from samples of fermenting egg white, are given in table 4.

From table 4 it can be seen that there were quite a diverse number of species of the genus *Pseudomonas* encountered. The strains listed represent only those isolations made at the conclusion of the periods employed in experimental fermentations. If the isolations made from all lots of freshly broken-out egg whites studied had been listed here, the diversification of species would have reached formidable proportions. Species of this genus seem to be very prevalent in egg

TABLE 4

Serratia and Pseudomonas group, gram-negative motile rods (usually occurring singly) or cocco-bacteria, indole negative

PROBABLE SEECIES	AGAR SLANT	GELATIN STAB.	LITMUS MILK	NITRATE REDUCTION	GLUCOSE BROTH	POTATO SLANT	FLUORESCENCE IN ULTRA-VIOLET LIGHT WHEN GROWN IN STERILE EGG WHITE
Serratia marcescens* (2-cultures)	2 bright fucsin color	2 infundibuliform liquefaction	2 acid curd	2+	2+ (acid but no gas)	Profuse dark red	2−
Pseudomonas ovalis† (2-cultures)	1 greyish white, 1 greenish white	2 no liquefaction	2 slight alkaline curd	2−	2−	1 dirty white 1 yellow to brown	1+ (blue), 1+ (blue green)
Pseudomonas fluorescens† (2-cultures)	2 olive green to white	2 infundibuliform liquefaction	2 slight alkaline curd	2+	2+ (acid but no gas)	1 yellow to brown, 1 dirty cream	2+ (bright green)
Pseudomonas aeruginosa† (1-culture)	Olive green	Infundibuliform liquefaction	Acid curd peptonized	+	+ (acid but no gas)	Yellow	+ (bright green)
Pseudomonas jaegeri† (1-culture)	Greenish white	Saccate liquefaction	Alkaline curd peptonized	−	+ (acid but no gas)	Brown to orange	+ (bright green)
Pseudomonas chlororaphis† (1-culture)	Greenish white	No liquefaction	Coagulated with green pellicle	+	Pigment crystals produced	Brown to orange	+ (bright green)
Pseudomonas schuylkilliensis† (1-culture)	Bluish white	Stratiform liquefaction	Alkaline curd peptonized	−	+ (acid but no gas)	Orange to brown	+ (blue green)

* Peritrichous flagella with Gray's stain.
† Polar flagella with Gray's stain.

white separated from storage eggs and a description of the various species encountered appears to be a task far too large to be included herein.

Since it is quite easy, with freshly laid eggs, to obtain sterile egg white in any quantity desired, if asceptic technique is employed in breaking the shell and separating the white from the yolk, a study was made to determine the changes in sterile egg white brought about by representative strains of the groups of cultures listed in tables 1, 2, 3, and 4.

These fermentations were carried out with 250 ml. quantities of sterile egg white in individual sterile glass jars of 500 ml. capacity. Inoculations were made from 48-hour starter cultures growing in sterile tubes of egg white at 30°C. Di-

lution plate counts were made on the starter cultures at the time of inoculation, using glucose agar. By using this count and the volumes of starter added, it is possible to calculate the approximate number of bacterial cells per ml. at the beginning of the fermentation period. This, of course, makes it possible to determine the influence of the size of the inoculum on the rate of fermentation by any selected strain of bacteria.

Using a selected strain of *Aerobacter aerogenes* five different volumes of inocula were employed and samples were removed aseptically at periodic intervals during 168 hours of incubation at 30°C. for pH measurements, sugar determinations by the method of Stiles, Peterson and Fred (1926) and formol titrations for combined amide and amino nitrogen.

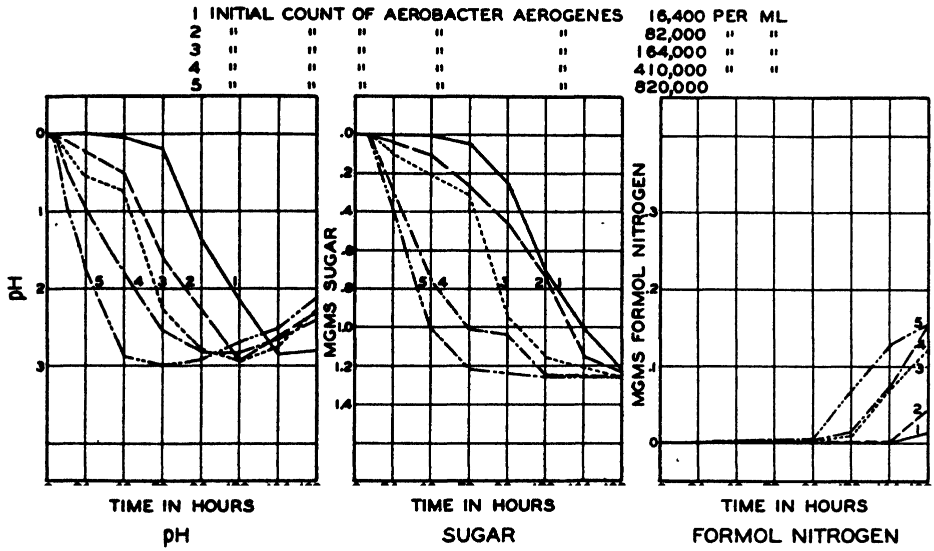

FIG. 1. CHANGES OVER THE INCUBATED STERILE CONTROL IN EGG WHITE INOCULATED WITH VARYING NUMBERS OF CELLS OF AEROBACTER AEROGENES

The rate and extent of change in pH, sugar, and formol nitrogen in these inoculated samples was determined by comparing the values found in these determinations at the different time intervals with the values obtained at the corresponding time intervals for incubated sterile control samples. The calculated deviations from the control are presented graphically in figure 1, in which values for the control are indicated by 0.

From figure 1 it can be seen that the rate of change in pH is directly proportional to the number of cells of *Aerobacter aerogenes* present at the beginning of the incubation period. With an initial count of 820,000 cells per ml., there is a change in pH of 3.0 in 72 hours; with 410,000 cells per ml., 96 hours are required for a change of a comparable size; with 164,000 cells 120 hours are required; with 82,000 cells 120 hours; and with 16,400 cells 144 hours. There is a subsequent change in the opposite direction in pH with each inoculated sample and the

amount of this change at the 168-hour interval appears also to be directly proportional to the number of cells initially present and thus directly proportional to the initial rate of change in pH.

The changes in sugar content over the uninoculated control parallel very closely the changes in pH with each sample. Thus, the rate of sugar utilization is also directly proportional to the number of bacteria initially present. That is, the larger the inoculum, the more rapid the utilization of the available sugar. The term "available sugar" is used here since it would appear that not all of the sugar is removed by the bacteria. The amount of sugar appears to decrease only to a relatively low constant level.

No changes in the formol nitrogen titration values over the sterile incubated control sample were found during the initial stages of fermentation. However, changes did occur in the latter stages of the fermentations and the extent of these appears to be directly proportional to the increase in the pH values after the available sugar had been utilized. Thus, it appears that the amount of change in the combined amide and amino nitrogen in 168 hours is directly related to the initial rate of fermentation.

Practically no measurable changes occurred in the sterile control egg white incubated for 168 hours. Dilution plates made from this control white at the 168-hour period showed that some slight contamination had occurred during sampling since there was a count of 213 bacteria per ml. at that time. This figure is the average figure for three one-ml. platings. It would appear, however, that this contamination was not great enough to have had any appreciable biochemical significance.

A study similar to the one just described was made to determine comparatively the magnitude and rate of change in sterile egg white brought about by substantially the same numbers of cells of *Aerobacter aerogenes*, *Escherichia freundii*, *Serratia marcescens*, *Proteus sp.* and *Pseudomonas aeruginosa* when added in pure culture.

By comparing the values obtained with the inoculated samples of egg white for pH, sugar and formol nitrogen with those found for sterile, uninoculated, incubated control egg white, comparative values were obtained for the five organisms with regard to the extent and rate of change brought about in the egg white. The deviations from the control are presented graphically in figure 2, for each organism in which values for the control are indicated by 0.

From figure 2 it can be seen that the number of cells present at the beginning of the fermentation period was, within the range of experimental error in dilution plate counting, the same for all of the five species of bacteria employed in this study.

From this figure it is apparent that the change in pH with *Escherichia freundii* is somewhat greater and more rapid than with *Aerobacter aerogenes*. The extreme change produced (in 96 hours) tended to persist longer in the case of *Escherichia freundii* than in the case of *Aerobacter aerogenes*. Sugar utilization on the other hand appeared to be as great although somewhat slower with *Aerobacter aerogenes* than with *Escherichia freundii*. It is probable, therefore, that more of the sugar was converted into gas with the former organism than with the latter. There

was no significant change in the formol nitrogen values with either of these organisms within 120 hours.

With the other three organisms the pH changes over the control sample were neither so rapid nor so great as those brought about by *Aerobacter aerogenes* and *Escherichia freundii*. Sugar utilization was not so complete nor so rapid. There were pronounced changes in the formol nitrogen values as early as 24 hours with *Serratia marcescens*, 48 hours with *Proteus sp.* and 48 hours with *Pseudomonas aeruginosa*. With *Serratia marcescens* and *Proteus sp.* the amounts of combined amide and amino nitrogen continued to increase rapidly and were apparently still on the increase at the conclusion of the incubation period, or at 120 hours. However, with *Pseudomonas aeruginosa* the formol nitrogen value increased up to the 72-hour period and apparently remained constant thereafter.

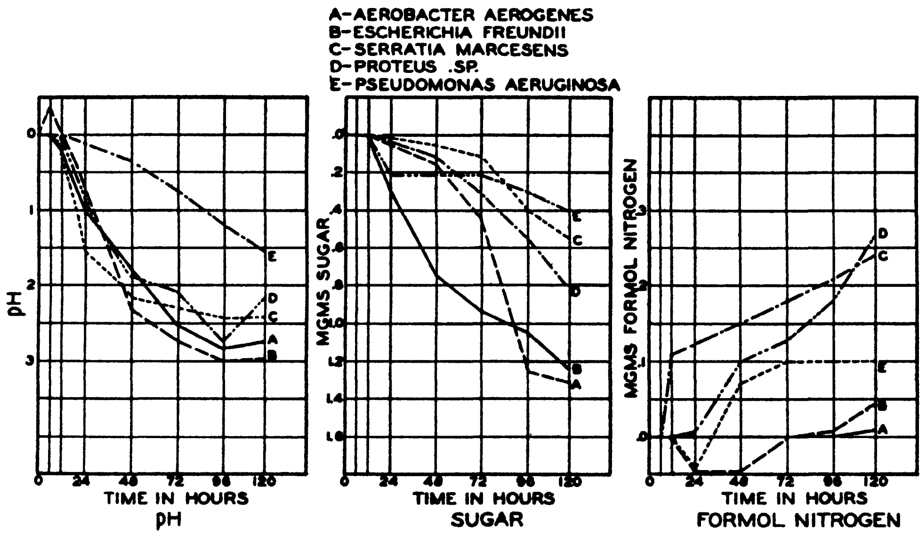

FIG. 2. CHANGES OVER THE INCUBATED STERILE CONTROL IN EGG WHITE INOCULATED WITH COMPARABLE NUMBERS OF BACTERIAL CELLS OF VARIOUS BACTERIAL SPECIES

The changes in pH and sugar values and formol titrations with egg white brought about by pure cultures of *Aerobacter aerogenes* and *Escherichia freundii* are the same as those found previously for naturally fermenting egg white. There can be no doubt, therefore, that these are the important organisms from the standpoint of normal, natural commercial fermentations.

SUMMARY

1. Twenty strains of bacteria isolated from fermenting egg white and previously identified as belonging to the genus *Aerobacter* were studied with the objective of establishing species identity. Twelve of these 20 isolations have been shown to be strains of *Aerobacter aerogenes*. The remaining eight strains were found to resemble *Aerobacter cloacae* more closely than *Aerobacter aerogenes* although they do not conform exactly with the descriptions given for either species.

2. Thirteen isolations from fermenting egg white previously identified as

belonging to the genus *Escherichia* have been shown through similar studies to be strains of *Escherichia freundii*.

3. Five isolations tentatively classified as belonging to the genus *Proteus* have not been identified with any recognized species since the results of microscopic and cultural studies do not clearly conform with the characteristics listed for recognized species.

4. Two red chromogenic isolations were studied and found to be strains of *Serratia marcescens*.

5. Eight isolations tentatively classified as belonging to the genus *Pseudomonas* have been studied and given tentative species identifications. Two were identified as strains of *Pseudomonas ovalis*, two as strains of *Pseudomonas fluorescens*, one as *Pseudomonas aeruginosa*, another as *Pseudomonas jaegeri*, another as *Pseudomonas chlororaphis* and the other as *Pseudomonas schuylkillensis*.

6. Lots of sterile egg white inoculated with varying numbers of cells of a selected strain of *Aerobacter aerogenes* fermented similarly to natural normal fermentations and the rate of fermentation was directly proportional to the number of bacterial cells added in the inoculum. That is, as the size of the inoculum increased, so did the rate of fermentation. In this study the course of fermentation was followed by pH measurement, sugar determinations and formol titrations, made at periodic intervals of time.

7. Sterile egg white inoculated with a selected strain of *Escherichia freundii* fermented in the same manner as sterile egg white fermented with *Aerobacter aerogenes*. The minor differences observed were a tendency for *Escherichia freundii* to produce acid more rapidly and maintain a lower pH longer than *Aerobacter aerogenes*.

8. Sterile egg white samples inoculated with selected strains of *Serratia marcescens*, *Proteus sp.*, and *Pseudomonas aeruginosa* and allowed to ferment did not show changes in pH, sugar content and formol titration values corresponding to normal natural fermentations. Decreases in pH and sugar content were neither so great nor so rapid as with *Aerobacter aerogenes* and *Escherichia freundii*. On the other hand, the fermentations with all three of these organisms were characterized by rapid and marked increases in the amount of formol nitrogen, indicating strong proteolytic action on the part of these species.

ACKNOWLEDGMENT

The authors are indebted to Dr. G. G. Slocum of the Food and Drug Administration, Federal Security Agency, for assistance in the species identification of the cultures reported.

REFERENCES

Bergey, D. H., R. S. Breed, E. G. D. Murray and A. P. Hitchens. 1939 Bergey's Manual of Determinative Bacteriology. 5th Ed. The Williams & Wilkins Co.

Parr, L. W. 1938 Coliform intermediates in human feces. J. Bact., 36, 1–15.

Stiles, N. R., W. H. Peterson and E. B. Fred. 1926 A rapid method for determination of sugar in bacterial cultures. J. Bact., 12, 427–434.

Stuart, L. S., and H. E. Goresline. 1942 Bacteriological studies on the "natural" fermentation process of preparing egg white for drying. J. Bact., 44, 541–549.

PSEUDOMONAS AERUGINOSA; ITS ROLE AS A PLANT PATHOGEN[1]

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The idea that some organisms may have the ability to establish themselves and thrive within both plant and warm-blooded-animal tissues has received the attention of comparatively few workers. The vast gulf between the two forms of life, in structure, composition, and many environmental factors, has seemed to preclude the thought that both could be favorable hosts to the same organism. Nevertheless, attempts have been made to show that such a dual pathogenicity can occur.

The most striking results were obtained by Benham and Kester (1932) using the fungus *Sporotrichum*. Employing strains isolated from both animals and plants, they found a few which would attack members of both kingdoms. The organism, *S. schenckii*, causing the human disease was transmitted to carnation and rose buds producing a rot similar to that caused by *S. poae*. After living saprophytically or parasitically in plants, *S. schenckii* retained its virulence for animals. In order to insure infection, however, it was necessary to provoke some slight injury before or at the time of the fungus injection. Ciferri and Baldacci (1934) inoculated 22 human pathogenic fungi and one insectivorous fungus into tomato fruits and found 18 that gave positive infection. They considered that their work not only confirmed that of Benham and Kester, but furnished additional evidence as regards the adaptability of human pathogens to plant hosts.

With bacterial pathogens less positive results have been obtained. Baldacci and Ciferri (1934) found two of 23 organisms of human source able to produce some evidence of infection in tomato fruits. These organisms were *Proteus vulgaris* and *Bacillus pyocyaneus*. In neither case was the infection severe, nor did it approach the type usually associated with the apical rot of this fruit.

Pseudomonas aeruginosa (*B. pyocyaneus*) has been a recognized human pathogen for a good many years. Its most distinguishing feature is the formation of the diffusible, chloroform-soluble, blue pigment, pyocyanin. This organism is widely distributed in nature, usually as a harmless saprophyte. On occasion, however, it can become a dangerous pathogen. Generally, it appears as a secondary invader and is often associated with suppurative lesions of various parts of the body. There are, nevertheless, numerous instances wherein it has been shown to be the primary cause of a fatal infection in man and other animals. Experimentally many domestic animals, rabbits, goats, mice, and guinea pigs are susceptible to infection. The animal pathogenicity of the organism is, therefore, a well established fact.

[1] This paper was presented in part on December 29, 1941, before the Society of American Bacteriologists in Baltimore.

Scattered throughout the literature are reports concerning the pathogenicity of *P. aeruginosa* for plants. For the most part, these accounts have not consisted in a systematic attempt to prove the actual phytopathogenicity of the organism. Rather, they are instances concerning the isolation of the bacterium from plant pathological conditions.

Verona and Passinetti (1926) obtained slight infection with *B. pyocyaneus* and *Bacillus fluorescens-liquefaciens* in lettuce. In each case, however, infection occurred only in plants injured by frost. Paine and Branfoot (1924) described a disease of lettuce caused by a bacterium which they concluded was identical with *Bacterium marginale*. A short time later, in the same laboratory, Mehta and Berridge (1924) pointed out that on the basis of morphological and cultural characteristics *B. marginale* was identical with *B. pyocyaneus*. The latter was shown to be capable of attacking young lettuce leaves and producing disease similar to that due to *B. marginale*.

Brooks, Nain, and Rhodes (1925) utilized the *B. marginale* culture obtained from Mehta in a serological comparison with *B. pyocyaneus* isolates of animal origin. In 5 anti-*pyocyaneus* sera *B. marginale* failed to agglutinate, nor did any of the 11 *B. pyocyaneus* organisms agglutinate in the *B. marginale* antiserum.

Desai (1935) described an organism producing a soft-rot of sugar cane which caused extensive damage in India. To this organism he gave the name "*B. pyocyaneus saccharum*" (*Phytomonas dexaioma*) and pointed out its apparent close relationship to *B. pyocyaneus*. He found that his organism was associated with a saprophyte and that the two organisms together produced greater infection than the pathogen itself.

In this country Clara (1934) included a single isolate of *B. pyocyaneus* in his study of the green-fluorescent plant pathogens. This culture did not prove to be pathogenic for the plants tested.

It is evident that the phytopathogenic nature of *Pseudomonas aeruginosa* has not previously been well established. However, our concern in the problem was initiated indirectly. In the course of a serological study of the green-fluorescent group of bacterial plant pathogens, it was found that one organism, *Phytomonas polycolor*, was extremely virulent for small laboratory animals (Elrod and Braun, 1941). The suspicion arose at that time that the bacterium we were dealing with was in reality *P. aeruginosa*. Subsequent experiments reported here have shown this to be true. With this fact established, we then believed it expedient to reinvestigate the phytopathogenic potentialities of *P. aeruginosa*. This report concerns such experiments, as well as those which demonstrated *P. polycolor* to be identical with *P. aeruginosa*.

CULTURES EMPLOYED[2]

The *P. aeruginosa* cultures used in this work were selected as representing a wide range of original habitats. Emphasis has been placed on cultures derived from animal sources, either normal or pathological.

[2] The writers are indebted to Dr. W. H. Burkholder, Miss Helen Knott, and Mr. Ed. Adams for supplying some of the cultures used.

G11, received as *P. polycolor*. The animal pathogenicity of this isolate was thoroughly investigated (Elrod and Braun, 1941). Produces an abundance of pyocyanin on glycerol-peptone agar after several days' incubation.

PP2, received as *P. polycolor*. Produces only small amounts of pigment at first; this property greatly enhanced by growth on glycerol-peptone agar.

Those known to be *P. aeruginosa* were:

Rab., isolated from a lung abscess in rabbit. Produces an abundance of pyocyanin on virtually all media.

Ky., stock culture from University of Kentucky. Atypical in that pyocyanin formation is overshadowed by the production of a brownish-black pigment.

Chick., isolated from the ovary of a chicken with a primary *Salmonella pullorum* infection. A strong pigment producer.

W., from intestinal tract of a normal man. A strong pyocyanin former.

Chi., originally from stock collected of University of Chicago *via* Ohio State University. Produces a light emerald green pigment.

OSU, stock culture from Ohio State University. A mediocre pyocyanin producer.

Birk., isolated from a middle-ear infection. A brilliant pigmenter.

RIH, isolated and suspected in gastro-enteric disturbance. Produces a large amount of pyocyanin.

A1, *A2*, *A3*, all isolated from water. Form deep blue pigment on virtually all media.

97, *256*, *257*, *260*, originally from the A.T.C.C. all derived from pathological lesions. These cultures produce no pyocyanin and but little fluorescin.

Our 17 isolates were all fatal to mice by intraperitoneal injection. Usually .05 ml. of an 18-hour broth culture was sufficient to kill the mouse in 12 hours. Those tested against rabbits and guinea pigs also proved to be fatal. In each case a bacteremia resulted and the organism was always cultivable in pure culture from the heart's blood. Previous experiments (Elrod and Braun, 1941) have shown that there is a definite multiplication of *P. polycolor* in the infected animals.

IDENTITY OF PHYTOMONAS POLYCOLOR WITH PSEUDOMONAS AERUGINOSA

P. polycolor was isolated and described by Clara (1930) as being the etiological agent of an economically important disease of tobacco prevalent in the Philippines. In the field and experimentally the organism produced a severe leaf spotting and necrosis, while in the seed beds it very often manifested itself as a soft-rot in the stems of young plants.

This species differs in many respects from the ordinary members of the green-fluorescent group of phytopathogenic bacteria. The two cultures in our possession (G11 and PP2) produce large amounts of pyocyanin. The pyocyanin is easily extracted after several days' incubation from either broth or agar slope cultures with chloroform. Pyocyanin formation is greatly enhanced by growing the culture on glycerol-peptone agar. Clara (1930), in his original description of the organism, noted this blue coloration on glycerol agar, but in a later comparative study (Clara, 1934) of certain members of the plant group failed to

isolate pyocyanin. According to Gessard, who is supported in this by Meader, Robinson, and Leonard (1925), the presence of pyocyanin is sufficient to identify an organism as *B. pyocyaneus*. Clara noted, as we have, that the organism grows more luxuriantly at 37°C. than at lower temperatures.

In an attempt to understand more fully the relationship between *P. polycolor* and *P. aeruginosa*, biochemical and serological studies were made. The carbohydrates used in the fermentation studies were incorporated in a synthetic medium to prevent any obliteration of acid formation by these strongly proteolytic organisms. This medium was made up of 0.2 g. magnesium sulfate, 0.1 g. calcium chloride, 0.2 g. sodium chloride and 0.2 g. dipotassium phosphate per liter. Incubation was conducted at both 37°C. and at room temperature.

TABLE 1

Biochemical reactions of Phytomonas polycolor and Pseudomonas aeruginosa

ORGANISM TESTED	GLUCOSE	SUCROSE	MANNITOL	GLYCEROL	SALICIN	RAFFINOSE	XYLOSE	ARABINOSE	MALTOSE	INDOLE	GELATIN
G11	A	–	–	–	–	–	A	A	–	–	+
PP2	A	–	–	–	–	–	A	A	–	–	+
Rab	A	–	–	–	–	–	A	A	–	–	+
Birk	A	–	–	–	–	–	A	–	–	–	+
Chick	–	–	–	–	–	–	A	–	–	–	+
97	A	–	–	–	–	–	A	A	–	–	+
260	–	–	–	–	–	–	A	A	–	–	+
257	A	–	–	–	–	–	A	A	–	–	+
256	A	–	–	–	–	–	A	–	–	–	+
W	A	–	–	–	–	–	A	A	–	–	+
A1	A	–	–	–	–	–	A	–	–	–	+
A2	A	–	–	–	–	–	A	A	–	–	+
A3	–	–	–	–	–	–	A	A	–	–	+
Chi	A	–	–	–	–	–	A	A	–	–	+
OSU	A	–	–	–	–	–	A	A	–	–	+
Ky	A	–	–	–	–	–	A	A	–	–	+
RIH	A	–	–	–	–	–	A	A	–	–	+

A, acid formed; –, no visible change; +, gelatin liquefied.

As one can see in table 1, glucose, xylose, and arabinose were fermented with the formation of acid only by almost all of the organisms. Sucrose, mannitol, maltose, glycerol, salicin, and raffinose were not acted upon. Indole was not formed, but gelatin was rapidly liquefied. The latter two reactions are considered characteristic of *P. aeruginosa*.

There is considerable difference of opinion among various authorities as to the actual fermentative capacity of *P. aeruginosa*. Moltke (1927), using 4 isolates, concluded that none of the more common carbohydrates were fermented; Bergey *et al.* (1939) evidently adhere to this belief. Other writers, Zinsser and Bayne-Jones (1939), Topley and Wilson (1931), and Sandiford (1937) agree that glucose is attacked. Whatever the fermentative abilities of the bacterium, it must be admitted that our 15 isolates of *P. aeruginosa* and 2 of *P. polycolor* are

in close agreement. Clara (1934) found his isolate of *P. polycolor* far more saccharolytic than is shown by our studies. According to him, glucose, galactose, levulose, mannose, arabinose, xylose, mannitol, and glycerol were all fermented. At the same time, however, the *P. aeruginosa* culture he utilized fermented all of these carbohydrates, as well as salicin. In the original description of the organism (Clara, 1930) xylose, arabinose, glucose, and mannose were said to be the only sugars fermented. This is similar to the results reported here.

The antigenic characteristics of *P. aeruginosa* are likewise in a state of confusion. It is generally agreed by most authorities that the species is serologically heterogeneous. Aoki (1926) using 50 isolates concluded, on the basis of agglutination tests, that the organisms were antigenically dissimilar. He employed 37 antisera and found that 22 separate groups were formed; some of these groups had but a single member, the largest 10.

TABLE 2

Agglutination tests

ORGANISM AGGLUTINATED	SERA PREPARED AGAINST:			
	Birk.	257	G11	Rab.
Birk.	3200*	<100	<100	<100
A2	3200	<100	<100	<100
Chick	3200	<100	<100	<100
A3	3200	<100	<100	<100
W	3200	<100	<100	<100
A1	1600	<100	<100	<100
OSU	1600	<100	<100	<100
RIH	<100	<100	<100	<100
257	<100	3200	25600	25600
260	<100	3200	25600	12800
PP2	<100	1600	25600	12800
G11	<100	1600	25600	25600
97	<100	3200	25600	25600
Chi	<100	3200	25600	25600
Rab	<100	3200	25600	25600
256	<100	3200	25600	25600
Ky	<100	<100	12800	6400

* Titers indicated as reciprocals.

Meader, Robinson, and Leonard (1925), by means of agglutinin-adsorption, stated that the *B. pyocyaneus* group was serologically uniform. They found wide variations in agglutinability in different sera, but adsorption with any of the isolates brought about complete reduction in both homologous and heterologous reactions.

In our study antisera were prepared against one isolate of *P. polycolor* (G11) and 3 of *P. aeruginosa* (Birk., Rab. and 257). Agglutination tests were performed with these 4 immune sera and the 17 isolates available. It is to be observed in table 2 that there are two large groups demonstrable. Included in

the first group are those organisms which agglutinate in only anti-Birk. serum, but in none of the other three sera. This group includes: A2, OSU, Chick., A3, W, A1, and Birk. In the second group are those organisms which failed to agglutinate in Birk. antiserum but agglutinated in each of the other three sera. Rab., 260, 97, Chi., 256, 257, G11, and PP2 comprise this group. Only two organisms fall outside of these two groups. The isolate RIH seems more closely allied to the first inasmuch as it failed to agglutinate in any of the sera, whereas Ky. appears closer to the second as it agglutinated strongly in both G11 and Rab. antisera but not in 257 antiserum. In each case the heterologous reactions were as strong, or nearly so, as the homologous. Complement fixation tests have confirmed every detail of the agglutination experiments.

TABLE 3

Agglutinin-adsorption experiments

ORGANISM AGGLUTINATED	G11 ANTISERUM ADSORBED WITH				257 ANTISERUM ADSORBED WITH			RAB. ANTISERUM ADSORBED WITH				BIRK. ANTISERUM ADSORBED WITH		
	Rab.	257	PP2	Birk	G11	PP2	Birk.	G11	257	PP2	Birk.	G11	257	Chick.
257	<100	<100	<100	25600	400	800	3200	<100	<100	<100	25600	*		
260	<100	<100	<100	25600	400	800	3200	<100	<100	<100	12800			
PP2	<100	1600	<100	25600	<100	<100	1600	<100	3200	<100	12800			
G11	<100	3200	<100	25600	<100	<100	1600	<100	3200	<100	25600			
97	<100	<100	<100	25600	800	800	1600	<100	<100	<100	25600			
Chi	<100	3200	<100	25600	<100	<100	1600	<100	1600	<100	25600			
Rab	<100	3200	<100	25600	<100	<100	3200	<100	3200	<100	25600			
256	<100	<100	<100	25600	800	800	3200	<100	<100	<100	25600			
Ky	<100	1600	<100	1280				<100	1600	<100	6400			
Birk												3200	3200	<100
A2												3200	3200	<100
Chick.												3200	1600	<100
A3												1600	3200	<100
W												3200	3200	<100
A1												1600	1600	<100
OSU												1600	1600	<100
RIH														

* Blank = no agglutination.

Agglutinin-adsorption tests have revealed that certain isolates are antigenically identical and others apparently so, but have failed to confirm the opinion of Meader *et al.* (1925) that by this method the group is serologically homogeneous. The results of the adsorption experiments are found in table 3.

With the 4 prepared sera certain facts are evident. By reciprocal adsorption the *P. aeruginosa* culture, Rab., and the *P. polycolor* isolate, G11, are antigenically identical. Likewise, the other strain of *P. polycolor*, PP2, is serologically very close to these two organisms. Adsorption with this bacterium removes all the agglutinins from both G11 and Rab. antisera. That the G11, Rab., 257 agglutination group is very likely complex is demonstrated by the adsorption of G11 serum with 257 and the latter antiserum with G11. In each case there

is a severe reduction in the homologous titer, but some residual agglutinins are left. G11 and Rab. apparently have factors in excess of those common to 257, and, at the same time, 257 has factors in excess of the other two.

Adsorption with the non-agglutinating (in G11 and Rab. antisera) Birk. failed to remove any agglutinins, either homologous or heterologous. Likewise, adsorption of anti-Birk. serum by G11 and 257 did not reduce the titer for Birk. nor remove any of the heterologous agglutinins.

The above adsorption experiments add evidence to the facts obtained from the agglutination and complement fixation studies. By them it is possible to demonstrate common antigenic factors, and at the same time the two serological groups are further emphasized. It is apparent that one of the groups (G11 Rab., 257, etc.) can be split still further by means of adsorption. The same may be true of the Birk. group.

PHYTOPATHOGENICITY OF PSEUDOMONAS AERUGINOSA

Inasmuch as *P. polycolor* had been isolated from tobacco, this plant became the choice in testing the phytopathogenicity of our *P. aeruginosa* cultures. We have produced on tobacco all of the symptoms described by Clara with certain of the *P. aeruginosa* isolates, as well as the two alleged *P. polycolor* organisms. Koch's postulates can always be fulfilled in regard to these experiments. Clara (1934) with his one *B. pyocyaneus* strain failed to get infection on any of the plants he tested, including tobacco.

Inoculation of the plants by needle puncture (both stem and leaves), leaf smears, and spraying were all effective to varying degrees. The most favorable method was that of the smear. The application of a loopful of a fresh agar slope culture to the surface of the leaf produced severe necrosis (fig. 3), followed by the destruction of the whole leaf. This technique was efficacious 100 per cent of the time and with all 17 isolates. A culture of *P. fluorescens* produced feeble lesions (easily distinguished from those caused by *P. aeruginosa*) by the smear method, but none by any other means. This "blunderbuss" method probably is most effective due to the amount of the inoculum used and to the transfer of certain toxic metabolites. Needle puncture iuoculations into the leaves of young plants produced lesions in about 40 per cent of the cases. Usually only the highly pigmented forms were active by this method. Stem punctures brought about a destructive soft-rot and wilt (fig. 2), typical of that seen in the seed beds by Clara (1930). Here again the pigmented forms were the most potent.

Both smear and needle puncture inoculations, although accepted phytopathological techniques, are subject to criticism. In neither case can one be certain that the organisms are actually invasive. The inefficiency of the needle puncture technique compared with that of the smear method seems due both to the introduction of a smaller amount of inoculum and to a decrease of toxic products, both of which apparently aid in infection. The introduction of the inoculum by spraying obliterates these objections and at the same time serves as a better index of the invasiveness of the organism. In our hands (and in Clara's), however, spray inoculations have not proved especially effective. Nev-

ertheless, we have been able to produce the disease by this method (fig. 1). The efficiency of the method can be greatly increased by first water-soaking the leaves of the plant.[3] In such water-soaked tissues infection took place readily and there was an active multiplication and spread of the bacteria. This resulted after 3 or 4 days in large, brown, necrotic areas.

Clara clearly recognized the necessity of certain predisposing environmental factors before *P. polycolor* became excessively destructive. He was not able, however, to define these. We have found that a temperature of 22° to 25°C. (and probably higher), a highly humid atmosphere, as well as a water-soaked condition, are beneficial for the establishment and spread of the organisms in the tobacco plant. The phenomenon of water-soaking undoubtedly plays a major role in promoting a spread of the organisms under natural conditions. Braun and Johnson (1939) have observed extensive natural water-soaking due to internal pressures in tobacco plants grown in seed beds as well as in many other plant species during periods of high humidity and high soil moisture. Clayton (1936) has reported that water-soaking results under field conditions from the impact of hard-driven rain on the tobacco leaf surface. When these meteorological conditions prevail, an epiphytotic can easily result. All these conditions do occur in the Philippines where damage resulting from this disease has been most severe.

We have noted, as did Clara, that the lower leaves on the plant are the most easily infected; this is due in part to the greater ease with which these leaves become water-soaked. The disease in the field is probably spread by means of rain-splashed contaminated soil and plants. The susceptibility of the lower leaves aids, therefore, in the spread of the organism.

The linking of *B. marginale* to *B. pyocyaneus* by Mehta and Berridge (1924) prompted us to attempt infections of the plants attacked by *B. marginale* with our *P. aeruginosa* isolates. This organism was originally isolated by Brown (1918) from lettuce. It usually manifests itself by a marginal wilt of the leaves and a progressive soft-rot. Red spots and streaks, not usually necrotic, are often scattered over the leaves. According to Dowson (1941), this organism is also effective in causing storage rots, and he lists the organism with the *Erwinia carotovora* group in this respect. He found potato, onion, and cucumber to be effectively attacked.

Spray inoculations of lettuce by *P. aeruginosa* (certain isolates) produced the same type of rot and wilt that was held due to *B. marginale* (fig. 4). The reddish spots were readily produced. It is our opinion that the red coloration is due to the formation of acid pyocyanin. Here again the pigmented forms were most effective. A large number of the cultures produced a rot of potato, cucumber, and onion seemingly identical with that ascribed to *B. marginale*. In these experiments sterile slices of the above-mentioned vegetables were inoculated by

[3] Water-soaking refers to the injection or flooding of the intercellular spaces of plant tissues with water. This condition was induced artificially by us by either driving water into the stomata of plants with an atomizer or by the application of water under pressure to the root system or cut stem ends of tobacco plants.

smearing the culture. Incubation was carried out at room temperature for a week or more. The rot produced is very similar to that caused by *E. carotovora* but progresses more slowly. This action is very likely due to the liberation of protopectinase.

Not all of the 17 isolates affect the plants, nor are the effects always the same. For the most part the highly pigmented organisms are the most infective. However, pyocyanin isolated as the pure base has not evoked any of the symptoms described. A summary of the plant inoculations is found in table 4. It was also noted that, whereas rough forms of the various strains had lost their pathogenicity for animals, they had retained the ability of attacking plant tissues. Passage through ~~plants~~ animals did not alter the effectiveness of the phytopathogenic

TABLE 4

Plant inoculations

ORGANISM TESTED	TOBACCO			ONION	CUCUMBER	POTATO	LETTUCE
	Needle puncture	Smear	Spray				
G11	+	+	+	+	+	+	+
PP2	+	+		+	+	+	+
Rab	+	+	+	+	±	+	+
Birk	+	+	+	+	+	+	+
Chick	+	+	+	±	+	+	+
97	−	+	−	−	+	−	−
260	−	+	−	±	+	−	−
257	−	+	−				
256	−	+	−	+	+	−	−
W	+	+		+	±	+	+
A1		+			−		+
A2		+		+	+	+	
A3		+			+		+
Chi	+	+	+	+	+	+	−
OSU	+	+			+		−
Ky	+	+		−	−	+	−
RIH	+	+	+	+	+	+	+

+, good reaction; ±, weak reaction; −, no visible change; blank = not done.

powers of the organisms. Likewise, plant passage did not detract from the ability of the various isolates to infect animals.

DISCUSSION

From the results of the biochemical and serological tests it is evident that the two isolates of *P. polycolor* are indistinguishable from certain strains of *P. aeruginosa*. The heterogeneity of the antigenic character of the group and the minor inconsistencies in the fermentative abilities do not detract from this relationship. Also, the production of pyocyanin by the two plant cultures confirms still further their true identity. Likewise, the pathogenicity for small animals of *P. aeruginosa* is also shared by *P. polycolor*. From the above facts

it would be impossible to separate *P. polycolor* from *P. aeruginosa*, and we conclude that the two are identical.

The similarity between the plant and animal isolates is further emphasized by plant inoculations. Both produce similar pathological conditions on tobacco. The lesions produced by us experimentally are identical with those described by Clara as occurring naturally and produced in the laboratory by him with *P. polycolor*. The production of soft-rot of onions, cucumbers, and potatoes by our cultures links them with *B. marginale*. Mehta and Berridge (1924), however, have already pointed out the similarity of *B. pyocyaneus* and *B. marginale*. Their experiments, and ours, would indicate that the 3 organisms are the same. In view of the serological heterogeneity of the group, the agglutination results of Brooks, Nain, and Rhodes (1925) would not invalidate this relationship.

Infection of plants, as of animals, by *P. aeruginosa* occurs best in a weakened host. Thus, a storage rot must be considered as a passive pathogenic process, implying little more than the ability to grow and excrete certain products (in this case the enzyme protopectinase) for inducing the pathological condition. In actively growing plants, also, a weakened state is conducive to good infection. Water-soaking must be looked upon as a debilitory state. Likewise, excessively high temperature and high humidity contribute to a weakened condition. It must be remembered, too, that the less actively growing lower leaves are the most susceptible. Such debilitating conditions have their analogies in virtually all *P. aeruginosa* infections in animals, wherein the organism serves either as a secondary invader or attacks a previously weakened host, e.g., undernourished children.

Neither in its plant pathogenicity, nor in its animal infections, can *P. aeruginosa* be considered an aggressive pathogen. As we have pointed out, the amount of the inoculum must usually be excessive or the host weakened. But in plants, as well as in animals, when conditions are favorable, the disease can be acute and widespread. The presence of *P. aeruginosa* in the soil very likely serves as a continual reservoir of infection. The susceptibility of the lower leaves likewise would aid in spreading and maintaining the disease. Brown (1918) recognized *B. marginale* as a soil organism and was confident that infection does occur by contaminated soil. The marginal aspect of the disease in lettuce would seem to bear this out. Soil contamination could well account for the presence of the organism in storage rots.

It appears likely that the phytotoxic factors of the organism are not the same as the toxic substances that induce animal disease. This was emphasized by the action of rough variants which, though not fatal to animals, retained their pathogenicity for plants. To date we have not been able to isolate the phytotoxic factor. Patty (1921) made an interesting observation concerning the production of HCN by *B. pyocyaneus* in culture and in the animal body. If this material is produced in sufficient quantity, it might well account for the necrotic lesions in plants.

It has been our experience, and that of others, to lose rabbits occasionally during a course of immunization with phytobacteria. Such deaths are ap-

parently due to an accumulative toxemia, the animals rapidly losing weight and becoming less active. On autopsy the organisms are not recoverable. Such is not the case with *P. aeruginosa* where a fulminating bacteremia is produced; showing the ability to adapt conditions within the animal body. In the plant, also, the organism thrives and multiplies. Without doubt the resulting lesions and other evidences of destruction are due to the production of toxic factors. Nevertheless, this in no way detracts from the ability of the organisms to adapt themselves to the conditions as set up in the plant. We have, therefore, in *P. aeruginosa* a bacterium which serves as a dual pathogen, and which, we believe, is unique in the field of bacteriology.

SUMMARY

Two isolates of *Phytomonas polycolor* have been found to be indistinguishable from cultures of *Pseudomonas aeruginosa.*

On the basis of pyocyanin formation, growth at 37°C., and animal pathogenicity, they react as does *P. aeruginosa.* Agglutination, complement fixation, and agglutinin-adsorption experiments have shown the two plant organisms to be serologically identical with at least one animal isolate and closely allied to others. A brief biochemical comparison has also indicated singleness of the group.

We have failed to confirm the view of Meader *et al.* that by agglutinin-adsorption the *P. aeruginosa* group is serologically uniform.

Fifteen isolates of *P. aeruginosa* derived from many sources have shown the ability to attack tobacco. The type of lesions produced is identical with that produced by *P. polycolor.* Many of these organisms produce a soft-rot of vegetables like that ascribed to *Bacterium marginale.* We concur with Mehta and Berridge in the identity of *P. aeruginosa* and *B. marginale.*

The ability of *P. aeruginosa* to thrive in plant tissues as well as in warm-blooded animals makes it unique in the field of bacteriology.

REFERENCES

Aoki, K. 1926 Agglutinatorische Einteilung von Pyocyaneus-Bazillen, welche bei verschiedenen Menschenerkrankungen nachgewiesen wurden. Zentr. Bakt. Parasitenk. I, **98,** 186–195.

Baldacci, E., and Ciferri, R. 1934 Intorno alla pathogenicità di alcuni batterii dell'uomo per il frutto del Pomodoro. Boll. soc. ital. biol. sper., **9,** 197–200.

Benham, R. W., and Kester, B. 1932 Sporotrichosis, its transmission to plants and animals. J. Infectious Diseases, **50,** 437–458.

Bergey, D. H., Breed, R. S., and Murray, E. G. D. 1939 Manual of Determinative Bacteriology, 5th Ed., The Williams & Wilkins Co., Baltimore.

Braun, A. C., and Johnson, J. 1939 Natural water-soaking and bacterial infection. Phytopathology, **29,** 2–3.

Brooks, R. St. J., Nain, K., and Rhodes, Mabel 1925 The investigation of phytopathogenic bacteria by serological and biochemical methods. J. Path. Bact., **28,** 203–209.

Brown, N. A. 1918 Some bacterial diseases of lettuce. J. Agr. Research, **13,** 367–388.

Ciferri, R., and Baldacci, E. 1934 Intorno alla pathogenicità di alcuni miceti dell'uomo per il frutto del Pomodoro. Boll. soc. ital. biol. sper., **9,** 200–202.

Clara, F. M. 1930 A new bacterial leaf disease of tobacco in the Philippines. Phytopathology, **20**, 691–706.
Clara, F. M. 1934 A comparative study of the green-fluorescent bacterial plant pathogens. Cornell Univ. Agr. Expt. Sta. Mem., 159.
Clayton, E. E. 1936 Water-soaking of leaves in relation to development of the wildfire disease of tobacco. J. Agr. Research, **52**, 239–269.
Desai, S. V. 1935 Stinking rot of sugarcane. Indian J. Agr. Sci., **5**, 387–392.
Dowson, W. J. 1941 The identification of the bacteria commonly causing soft-rot in plants. Ann. Appl. Biol., **28**, 102–106.
Elrod, R. P., and Braun, A. C. 1941 A phytopathogenic bacterium fatal to laboratory animals. Science, **94**, 520–521.
Meader, P. D., Robinson, G. H., and Leonard, V. 1925 Pyorubrin, a red water soluble pigment characteristic of *B. pyocyaneus*. Am. J. Hyg., **5**, 682–708.
Mehta, M. M., and Berridge, E. M. 1924 Studies in bacteriosis. XII. *B. pyocyaneus* as a cause of disease in lettuce and the identity of *B. marginale* with this organism. Ann. Appl. Biol., **11**, 318–323.
Moltke, O. 1927 Contributions to the characterization and systematic classification of *Bact. proteus vulgaris* (Hauser), Mumksgaard, Copenhagen.
Paine, S. G., and Branfoot, J. M. 1924 Studies in bacteriosis. XI. A bacterial disease of lettuce. Ann. Appl. Biol., **11**, 312–317.
Patty, F. A. 1921 The production of hydrocyanic acid by *Bacillus pyocyaneus*. J. Infectious Diseases, **29**, 73–77.
Sandiford, B. R. 1937 Observations on *Ps. pyocyanea*. J. Path. Bact., **44**, 567–572.
Topley, W. W. C., and Wilson, G. S. 1931 The Principles of Bacteriology and Immunity, Edward Arnold and Co., London.
Verona, O., and Passinetti, P. 1926 Su di un diperimento della *Lactuca sativa* L. Boll. ist. agrar. Scandicci, **11**, 364–376.
Zinsser, H., and Bayne-Jones, S. 1939 A Textbook of Bacteriology, 8th Ed., D. Appleton-Century Co., New York.

PLATE I

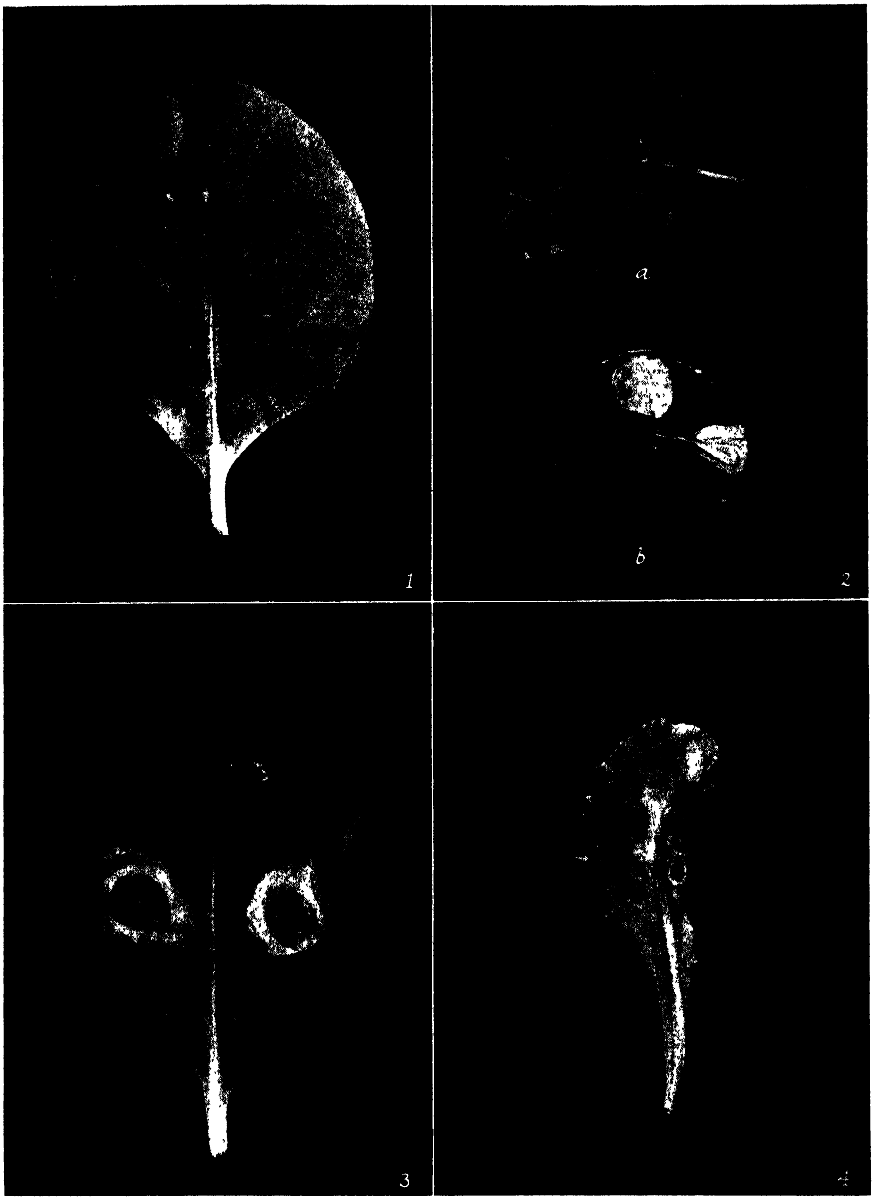
(Photographs by J. A. Carlile)

Fig. 1. A leaf of tobacco from a plant inoculated by spraying. Lesions are typical necrotic type. Culture Rab.

Fig. 2. a. Control tobacco plant inoculated into the stem with *Pseudomonas fluorescens*. b. Tobacco plant inoculated by stem puncture. Culture Birk.

Fig. 3. Tobacco plant inoculated by smear method. Culture 257.

Fig. 4. Leaf from lettuce plant inoculated by spray technique. Culture Chick.



(R. P. Elrod and Armin C. Braun: Pseudomonas Aeruginosa)

A STUDY OF TWO ATYPICAL STRAINS OF E. TYPHOSA

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In 1937, two atypical strains of *Eberthella typhosa* were isolated twenty-five days apart from patients presenting clinical symptoms of typhoid fever. Blood and feces from one patient, and blood from the other, yielded organisms atypical of *E. typhosa* in that they showed delayed fermentation of lactose and sucrose, but failed to produce hydrogen sulfide. Both patients gave a history of having eaten raw oysters at the same restaurant.

In reviewing the literature for reports of biochemically-atypical strains of *E. typhosa* one finds that as early as 1907 Twort artificially induced the fermentation of lactose by cultivation in lactose broth. This additional fermentative power, however, was quickly lost upon reinoculation to lactose-free media. Teague and Marchima (1920) studied particularly the variability of xylose and arabinose fermentation, and more recently Graf (1937) has strongly emphasized the frequency and extent of biochemical variation within the *Eberthella* group.

The discovery of biochemically irregular strains at primary isolation is, of course, not uncommon. Meyer and Neilson (1920) reported atypical reactions with dulcitol, rhamnose, milk and indole. Bacterial types intermediate between *Eberthella* and some other genus have also been reported. An example is that of Lazare and Breaks (1932) whose organism biochemically resembled the Hiss-Y type of dysentery bacillus, but possessed flagella and was agglutinated by both *E. typhosa* and *Shigella dysenteriae* antisera.

A case of temperorary variation following primary isolation is that of Poston (1938). Her strain fermented sucrose, but not lactose, and produced indole. It was at first weakly agglutinated (1:20) by typhoid antiserum, but became readily agglutinable at the same time that the biochemical reactions returned to normal. Poston's experience somewhat resembled ours in that culturally the atypical behavior was temporary and accompanied by partial inagglutinability. In our case, however, it was sucrose and lactose fermentation and hydrogen sulfide production which were abnormal.

Bergey's Manual (1939) lists three species[2] which resemble our isolations in that they ferment lactose and mannitol, but fail to liquefy gelatin. Each one of them, however, differs from our strains in at least one important respect. The sendai type of *E. typhosa*, like our strains, does not produce H_2S; but that organism differs in its fermentation of rhamnose and, of course, its inability to attack lactose or sucrose. To our knowledge the biochemical pattern herein described has not been reported previously.

[1] This paper is a summary of the senior author's Master of Science degree thesis.

[2] *Eberthella oxyphila, E. belfastiensis* and *E. pyogenes.*

EXPERIMENTAL

Description of organisms upon isolation. The cell morphology and smooth colonies were typical of *E. typhosa*. Few of the cells exhibited definite motility. Both strains (Jo and Tr) rapidly fermented glucose, maltose and mannitol. Xylose, arabinose, and salicin were negative. On the third day lactose and sucrose were acidified. Evidence of H_2S production was completely lacking; colonies on or in bismuth sulfite media (Difco) were uniformly colorless. Brom-cresol-purple milk was temporarily acid without coagulation, but later reverted

TABLE 1

Biochemical reactions of Jo and Tr strains five months after isolation

TEST SUBSTANCES	READINGS							
	Jo				Tr			
	1 day	3 days	7 days	14 days	1 day	3 days	7 days	14 days
Glucose	A	A	A	A	A	A	A	A
Maltose	A	A	A	A	A	A	A	A
Xylose	A	A	A	A	A	A	A	A
Galactose	A	A	A	A	A	A	A	A
Mannitol	A	A	A	A	A	A	A	A
Levulose	A	A	A	A	A	A	A	A
Lactose*	–	A	A	A*	–	A	A	A*
Sucrose*	–	A	A	A*	–	A	A	A*
Glycerol	–	–	A	A	–	–	A	A
Dulcitol	–	–	–	A	–	–	A	A
Trehalose	A	A	–	–	A	A	–	–
Sorbitol	A	A	–	–	A	A	–	–
Indole	–	–			–	–		
Nitrate reduction	+				+			
Hydrogen sulfide	–	–	–	–	–	–	–	–
Methyl red	+				+			
Voges-Proskauer	–				–			
B-C P milk	N	A	N	Alk	N	A	N	Alk
Gelatin	–	–	–	–	–	–	–	–

* The final pH of 1.0 per cent sucrose and lactose broths was 4.8 to 5.0.
A denotes acid, no gas. N denotes neutral reaction.

to slight alkalinity. Gelatin was not liquefied. Indole was not formed; nitrates were reduced.

Five months after isolation. The cultures had been transferred monthly on extract agar, incubated for 24 hours and stored at 4°C. Table 1 shows the results of biochemical tests at this time.

It will be noted from table 1 that both organisms were still atypical with respect to lactose, sucrose and hydrogen sulfide. Motility was now vigorous. Detection of hydrogen sulfide was attempted by the inoculation of the following media: lead acetate agar stabs; bismuth sulfite (Difco) medium, both as pour and streak plates; 0.03 per cent cysteine agar and broth. All were consistently negative, although the Rawlings strain (control) was uniformly positive. It

was further found that glycerol and dulcitol showed delayed fermentation. On the other hand arabinose, salicin, dextrin, inulin, cellobiose, inositol, rhamnose and raffinose were not attacked.

Eight months after isolation. Both strains had now lost the ability to ferment lactose, sucrose and dulcitol. Glycerol was still acidified. As yet neither strain produced H_2S; indeed, it was a little more than a year after isolation before these tests became positive. Both strains reverted to a typical biochemical pattern in about the same length of time.

Attempts to induce lactose fermentation. Since both strains had continued to attack lactose for at least five months after isolation, it seemed of interest to attempt reactivation of that enzyme. In spite of 19 months of bi-weekly subculturing in lactose broth at 37°C., both aerobically and anaerobically, lactose fermentation did not reappear. Believing that the utilization of lactose may originally have been associated with virulence, serial passage through mice was resorted to. Both strains killed mice regularly within 24 hours. Serial inoculations of peritoneal exudate were made at 24-hour intervals. After eighteen such passages lactose was not attacked, and further effort along this line was discontinued.

SEROLOGICAL STUDIES

Sera from patient Jo during the course of illness showed, as was expected, a gradual increase in somatic and flagellar typhoid antibodies. On the other hand, the second patient (Tr) during her entire hospital stay failed to develop a titer above 1:20 against typhoid-901 antigen.

Formalinized suspensions of the freshly-isolated strains were only partially agglutinated by high-titer OH-901 antiserum; Jo antigen was positive through 1:100 dilution, and the Tr suspension through 1:50 only. No agglutination of living bacilli by O-901 serum could be detected. Reactions with various *Salmonella* sera were minimal or entirely absent. Pure "Vi" serum was not available. The inhibition of flagellar agglutination was compatible with the few flagella seen by appropriate staining procedures. Absence of "O" agglutination, on the other hand, was interpreted as being due to the presence of considerable "Vi" antigen.

Two months after isolation both strains were readily agglutinated by "H" and "O" sera. It may be of interest to note that the biochemical reactions were still atypical. About this time rabbit antisera were produced with formalinized suspensions and the serological relationships with several *Salmonella* and *Shigella* species determined. Since originally both strains had shown feeble motility and delayed fermentation of lactose, it seemed advisable to test particularly for any antigenic relationship with *Shigella sonnei*. In brief, "H" and "O" reciprocal agglutination tests revealed the anticipated cross reactions with certain *Salmonella* species, but none with Shiga, Flexner or Sonne dysentery bacilli. By reciprocal absorption the serological similarity of cultures Jo and Tr with typhoid strain 901 was established. Therefore, we felt that at this time we were dealing with fully antigenic typhoid bacilli and no attempt was made to determine their antigenic structure or to type them by bacteriophage.

When the biochemical behavior of the strains eventually became typical, new antisera were prepared and the agglutination studies repeated. No essential differences were noted. Somewhat later it was decided to compare our strains with *Eberthella* sp. (Sendai type) which is also H_2S-negative, but ferments rhamnose. Sendai strain #6968 from the American Type Culture Collection was accordingly tested against the various Jo and Tr sera and against stock typhoid sera. No relationship could be found between the "H" agglutinogens of the sendai and several typhoid strains.

Studies on carrier state. Patient Jo, unfortunately, was not available for follow-up study, but the other patient (Tr) became a carrier and recently was still eliminating "*E. typhosa*" in the feces and urine. It may be of interest that the isolations obtained up to approximately one year after onset were biochemically identical with the original isolation but usually reverted quickly to the lactose-negative, sucrose-negative, H_2S-positive type. One year later both typical and atypical types were present. But when the cultures were again repeated after an additional six months, three biochemical types were recovered. In addition to the typical and atypical varieties previously encountered, there appeared a transitional H_2S-positive form fermenting sucrose, but not lactose. Three years after onset only typical organisms could be found.

Several times during the series of periodic feces and urine examinations blood was drawn from this individual and tested for antibody. It is of interest that at no time could a titer above 1:50 against the homologous or 901 typhoid antigens be demonstrated. In addition, each new isolation was for a time poorly agglutinated by typhoid "O" sera.

Both the partial inagglutinability of strain Tr at isolation and its persistence in the patient after clinical recovery suggested the presence of "VI" antigen. In fact, it was demonstrated as long as three years after isolation that both strains contained a small amount of this antigen. Their virulence for mice was approximately one-half that of the high-"Vi" Watson strain (Eliot, 1940), and following mouse passage living suspensions were agglutinated (1:320) by pure "Vi" antiserum. In addition, the patient's serum consistently agglutinated "Vi" antigen in 1:40 dilution.

DISCUSSION

The isolations described in this communication appear to represent a single variant type. Their origin was the same and their entire behavior during the period of study was remarkably similar.

Because H_2S was not produced upon primary isolation, neither strain could be detected in bismuth sulfite agar. It is not unlikely that similar variants occasionally occur among the H_2S-positive *Salmonella* group. Therefore, sole reliance upon this type of medium for the primary isolation of H_2S-positive enteric bacilli is undesirable.

That markedly atypical strains may cause the classical form of typhoid fever is illustrated by these two cases. Both patients presented the usual symptoms at onset and subsequently pursued the customary course of typhoid fever.

SUMMARY

1. Two biochemically atypical strains of *Eberthella typhosa* from clinical typhoid fever were observed for a period of three years.

2. Upon isolation lactose and sucrose were fermented, but H_2S was not produced. Only after one year of artificial cultivation did they become entirely typical.

3. All attempts to reinduce lactose fermentation failed.

4. Serologically both strains possessed considerable "Vi" antigen, but when isolated only a small amount of flagellar antigen. No other unusual antigenic property was observed.

5. Periodic feces isolations from one case, a carrier, revealed a gradual transition from the abnormal biochemical pattern first observed to a completely typical one three years later.

REFERENCES

Bergey, D. H., Breed, R. S., and Murray, E. G. D. 1939 Manual of Determinative Bacteriology. Fifth Edition, The Williams & Wilkins Company, Baltimore.

Eliot, C. P. 1940 Vi agglutination as an aid in the detection of chronic typhoid carriers. Am. J. Hyg., **31**, Sec. B, 8–15.

Graf, W. 1937 Untersuchungen über atypische Typhusstämme. Arch. Hyg., **119**, 145–152.

Lazere, A. H., and Breaks, V. 1932 An organism intermediate between *Bacillus typhosus* and *Bacillus dysenteriae Shiga* isolated in typical typhoid fever; a report of two cases. J. Infectious Diseases, **50**, 473–483.

Meyer, K. F., and Neilson, N. M. 1920 Irregular typhoid strains and the infections caused by them. J. Infectious Diseases, **27**, 46–71.

Poston, M. A. 1938 Atypical typhoid fever caused by atypical strains of *Eberthella*. J. Bact., **35**, 56–57.

Teague, O., and Marchima, K. 1920 Action of *B. typhosus* on xylose and some of the other less frequently used sugars. J. Infectious Diseases, **26**, 56–58.

Twort, F. W. 1907 The fermentation of glucosides by bacteria of the typhoid-coli group and the acquisition of new fermentative powers by *Bacillus dysenteriae* and other microorganisms. Proc. Roy. Soc. (London) B, **79**, 329–336.

SPORE FORMATION BY BACILLUS SUBTILIS IN PEPTONE SOLUTIONS ALTERED BY TREATMENT WITH ACTIVATED CHARCOAL[1]

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INTRODUCTION

Some years ago it was found that *Bacillus subtilis* sporulated more profusely in Bacto-peptone broth when a small quantity of activated charcoal was added to the medium. Further studies revealed that although the charcoal was removed from the medium prior to inoculation, the percentage of cells sporulating during short incubation periods was still appreciably greater than in untreated Bacto-peptone broth. The work subsequently done to determine the nature of the response of *B. subtilis* to charcoal treatment is the subject of the present report. Although the investigations are not completed, publication of the available data seems advisable since it is improbable that further work will be possible in the near future. Apparently the influence on sporulation of various fractions of commercial peptones has not previously been studied.

MATERIALS AND METHODS

Unless otherwise stated, Bacto-peptone solutions were used as basal media to which the various colloids under investigation were added. The nutrient solutions were placed in six-ounce prescription bottles, each containing 25 ml. of desired solution. The bottle screw-caps were carefully adjusted to prevent contamination without hindering diffusion of gases. In most cases the bottles were incubated in a horizontal position to allow a maximum of surface, since this treatment prevented heavy pellicle formation and facilitated accurate sampling. Cultures were incubated at 37°C.

The test organism used was a strain of *B. subtilis* originally obtained from the University of California Medical School. It is the culture used by Williams (1931) in his earlier studies on the physiology of sporulation. In most cases, the various nutrient solutions to be tested were inoculated with two drops of a suspension of cells of *B. subtilis*, obtained by washing a 24-hour 0.5 per cent Bacto-peptone agar slant culture with about 50 ml. of sterile distilled water.

Loop smears were made from the incubating cultures at intervals, usually after two and four days. Spores were stained by the method described by Conklin (1934) and spore-vegetative cell ratios were determined by counting 400–500 cells, distributed over five to ten microscopic fields.

[1] This paper covers in part the dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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EXPERIMENTAL

A. Relationship of sporulation to nutrient supply in peptone solutions treated with charcoal

Williams (1931), Brunstetter and Magoon (1932), and others have shown that sporulation in peptone solutions is inversely proportional to the concentration of the peptone solution. Adsorption of nutrients might explain increased sporulation in peptone solutions treated with charcoal. Preliminary studies indicated that 0.5 per cent Bacto-peptone solution contained approximately 0.75 mgm. of nitrogen per ml., whereas a 0.5 per cent solution heated with charcoal

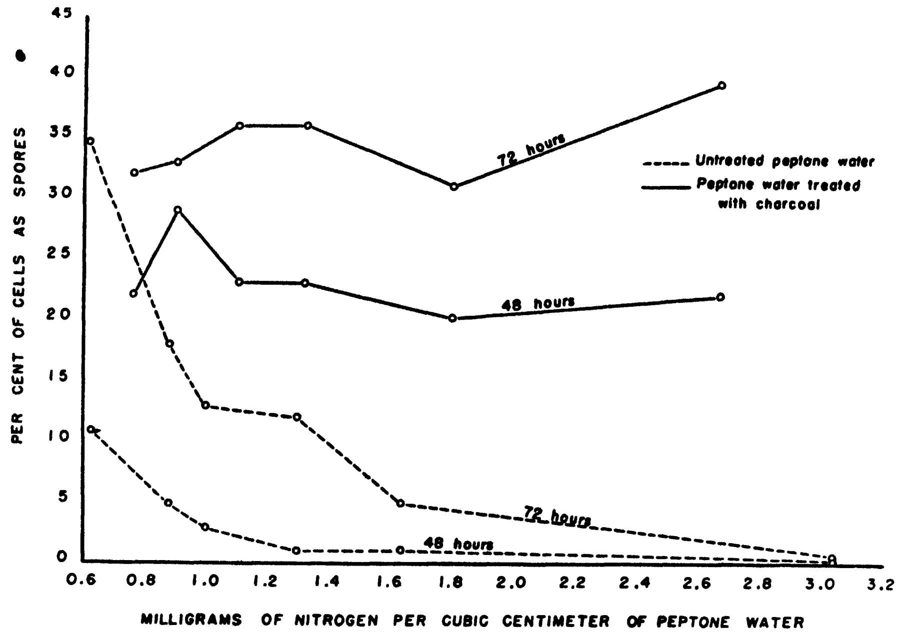

FIG. 1. SPORULATION BY B. SUBTILIS IN VARIOUS CONCENTRATIONS OF UNTREATED AND CHARCOAL-TREATED BACTO-PEPTONE WATER AFTER 48 AND 72 HOURS OF INCUBATION (Concentration estimated by total nitrogen content)

and filtered contained only about 0.19 mgm. of nitrogen per ml. Thus, if total nitrogen is a valid index of total nutrients, then approximately two-thirds of the nutrients are removed by treatment of the peptone solution with charcoal. The following experiment was done to determine if the action of charcoal in stimulating sporulation is due to a reduction in the amount of total available nutrients.

A one-per-cent Bacto-peptone solution was treated with two per cent Norite and repeatedly filtered with suction until the filtrate was free from charcoal. The pH of the peptone solution during adsorption was 6.5. The clear filtrate was then evaporated to approximately one-seventh of the original volume. At intervals during evaporation samples were removed from the evaporating dishes and the total nitrogen content of each determined. Each sample was then

inoculated with *B. subtilis* and incubated for 96 hours. After 48 and 96 hours incubation, counts were made to ascertain the per cent of cells in the spore state. The results are given in figure 1.

Treatment with charcoal improves the medium for sporulation regardless of the concentration of nutrients at which the comparison is made. Also spore formation in untreated peptone water has an inverse, curvilinear relationship to peptone concentration, whereas spore formation in treated peptone water is not appreciably influenced by the concentration of peptone as measured by total nitrogen. This evidence indicates that treatment with charcoal influences spore formation in some manner other than by reducing the concentration of total nutrients as measured by total nitrogen. However, it is quite possible that charcoal treatment so completely removes some essential nutrient factor that even after concentration of the filtrate this factor remains a limiting factor for continued vegetative development. It would seem that spore formation by *B. subtilis* in Bacto-peptone water is inversely proportional to the concentration of either a nutrient factor (not total nutrients) or some factor directly inhibitory to the spore-forming process.

TABLE 1

*Comparison of growth of B. subtilis in charcoal-treated and untreated bacto-peptone broth of equal total nitrogen content**

HOURS OF INCUBATION	MILLIONS OF CELLS PER ML.	
	Untreated	Charcoal-treated
1	.4	.5
2	1	.8
4	25	21
5	39	38
6	86	70
8	107	96
10	103	67
14	111	134
24	114	96
36	139	100
50	107	92
60	85	98
77	78	85
96	89	110

* 0.75 mgm. N per ml.

In order to obtain additional evidence relative to the comparative nutritive value of treated and untreated peptone waters of equal nitrogen content, growth of *B. subtilis* was measured in charcoal-treated and untreated peptone waters each containing 0.75 milligram of nitrogen per ml. The media were inoculated with approximately 430,000 cells per ml. of an 18-hour culture of *B. subtilis*. Increase in the number of cells was measured periodically by means of plate counts in the usual manner. The results are shown in table 1.

The untreated peptone water may have supported slightly better growth than treated peptone water of equal nitrogen content. The difference in growth rates in the two media is small and may be due only to inaccuracies in the plating technique. If treatment of Bacto-peptone with charcoal stimulates spore formation by the removal of essential food factors, then it is apparent that the removal of these factors has little, if any, influence on the total number of cells produced in the medium.

B. Some properties of the material adsorbed by charcoal

Occurrence. One-per-cent distilled-water solutions of Bacto, Witte, Parke-Davis, and Difco proteose peptones were subjected to adsorption by two per cent Norite without heat. Adsorption was done at the pH of the respective distilled water solutions. After filtration, each solution was evaporated until solutions equivalent in total nitrogen to 0.9, 0.8, 0.7, 0.6, and 0.5 per cent unadsorbed peptone solutions were obtained. A corresponding series of untreated solutions was prepared for each brand of peptone tested.

TABLE 2

Spore formation by B. subtilis in charcoal-treated solutions of various brands of peptone after 48 hours incubation

PEPTONE CONCENTRATION*	PER CENT OF CELLS AS SPORES IN INDICATED MEDIA							
	Charcoal treated				Untreated			
	Parke-Davis	Witte	Bacto	Proteose	Parke-Davis	Witte	Bacto	Proteose
per cent								
0.9	37	19	34	14	9	20	1	8
0.8	28	22	†	10	†	19	1	8
0.7	†	25	36	14	17	18	1	†
0.6	40	27	33	10	6	13	2	7
0.5	30	22	27	14	9	28	†	9

* Estimated by total nitrogen content in solutions treated with charcoal.

† Discarded because of evidence of contamination.

Bacto-peptone and Parke-Davis peptone solutions were better media for sporulation following partial adsorption by charcoal (table 2). At the pH of their respective distilled water solutions, Witte peptone and Proteose peptone respond only slightly if at all.

Adsorption with agents other than charcoal. Two-hundred-ml. aliquots of one per cent Bacto-peptone water at pH 6.5 were adsorbed with about two per cent of kaolin, ten ml. of aluminum hydroxide suspension containing approximately 16.1 mgm. of aluminum hydroxide per ml., and with ten ml. of ferric hydroxide containing about 20 mgm. of ferric hydroxide per ml. of suspension. Following adsorption and filtration, all the filtrates were adjusted to contain 0.77 mgm. of nitrogen per ml., the equivalent in total nitrogen of a 0.5 per cent untreated Bacto-peptone solution. These solutions were inoculated and incubated in the

usual manner. The results are presented in table 3. It will be noted that these materials, like charcoal, are effective in improving Bacto-peptone solution for sporulation by *B. subtilis.*

Hydrogen-ion concentration at which optimum adsorption with charcoal occurs. Aliquots of one-per-cent Bacto-peptone water were adjusted with HCl or NaOH to H-ion concentrations ranging from pH 3.0 to 10.0. One per cent Norite was added to each flask, heated, and filtered from the solutions. There was little change in pH during the adsorption.

Following removal of the charcoal, each solution was adjusted to pH 7.0 and total nitrogen was determined by Kjeldahl. All the solutions were then read-

TABLE 3

The influence on spore-formation by B. subtilis of treatment of bacto-peptone broth with adsorbents other than charcoal

ADSORBENT	PERCENTAGE SPORULATION IN MEDIA ADJUSTED TO CONTAIN 0.77 MGM. OF NITROGEN PER ML.	
	48 hours incubation	96 hours incubation
Control (not adsorbed)	2	23
Kaolin	26	52
Ferric hydroxide	29	59
Aluminum hydroxide	16	45

TABLE 4

Spore formation by B. subtilis in bacto-peptone water previously treated with charcoal at various hydrogen-ion concentrations

INCUBATION PERIODS	PER CENT SPORULATION IN PEPTONE WATER TREATED WITH CHARCOAL AT INDICATED pH*								0.5 PER CENT PEPTONE WATER UNTREATED
	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	
hours									
48	31	30	17	5	3	0	2	0	3
96	46	44	44	31	19	3	12	7	18

* After adsorption all media were adjusted by dilution to contain 0.77 mgm. of nitrogen per ml.

justed by dilution to contain approximately 0.77 mgm. of nitrogen per ml. The reduction in total nitrogen content of the peptone water was approximately the same regardless of the pH at which adsorption occurred. The percentage sporulation by *B. subtilis* in each medium is given in table 4. It is apparent that adsorption of Bacto-peptone broth within the pH range 3–6 produces the best medium for sporulation.

C. Comparison of the action of charcoal and agar on sporulation in Bacto-peptone water

It is well known that aerobic spore-forming bacteria will sporulate more profusely on the surface of media solidified by agar than in corresponding liquid

media. There is good evidence that the action of agar is not due entirely to improved oxygen relationships on the surface of the jel. For example, agar in concentration as low as 0.06 per cent has been observed to increase sporulation by *B. subtilis.* This concentration of agar imparts some viscosity but does not jel the medium. Also, the percentage sporulation in vigorously aerated Bacto-peptone broth has been found to be considerably less than in the unaerated medium made slightly viscous with agar. The per cent of cells as spores was determined from samples taken from below the surface of the medium. The ability to stimulate spore formation by *B. subtilis* is presumably a characteristic of many, if not all, hydrophilic colloids since gum karaya, gum tragocanth, gum arabic, quince seed extract, and purified *Phytomonas tumefaciens* gum have been observed to stimulate spore formation.

TABLE 5

Spore formation by B. subtilis as influenced by the addition of agar to various concentrations of charcoal-treated and untreated bacto-peptone broth

INCUBATION PERIOD	PEPTONE CONCENTRATION*	PER CENT OF CELLS AS SPORES IN PEPTONE WATER TREATED AS INDICATED			
		Charcoal-treated		Untreated	
		Agar 0.1 per cent	No agar	Agar 0.1 per cent	No agar
hours	*per cent*				
48	0.5	56	32	42	2
	0.8	52	27	38	1
	1	50	22	25	0
	2	51	33	2	0
	3	42	28	0	0
96	0.5	72	51	78	21
	0.8	63	60	68	5
	1	62	60	54	10
	2	75	76	28	3
	3	76	79	10	4

* In the case of charcoal-treated peptone solutions, the concentration is adjusted to correspond in total nitrogen per ml. to corresponding concentrations of untreated peptone water.

The following experiment was done to compare the influence of agar and charcoal on spore formation. Bacto-peptone water was treated with charcoal and evaporated until the total nitrogen content of the concentrate was equal to that of a 3 per cent untreated peptone water. By dilution of this concentrate, solutions were prepared which corresponded to two, one, 0.8, and 0.5 per cent peptone water. A corresponding series of untreated peptone solutions was also made. One-tenth of one per cent of agar was added to 25 ml. aliquots of each concentration of both treated and untreated peptone solutions. A corresponding series received no agar. The percentage sporulation by *B. subtilis* was measured after 48 and 96 hours of incubation. The results are shown in table 5.

It is at once apparent that the actions of agar and of charcoal are quite dissimilar. Agar stimulates spore formation in peptone water treated with charcoal and unlike charcoal has no influence in overcoming the deterrent effect of high concentrations of peptone.

SUMMARY

A greater percentage of *Bacillus subtilis* cells sporulate in Bacto-peptone broth treated with charcoal than in untreated peptone water.

The per cent of cells in the spore state is independent of the concentration of the treated peptone solution. It is suggested that spore formation in Bacto-peptone water is inversely proportional either to some particular food factor or factors or to some factor directly inhibitory to the spore-forming process.

Treatment of Bacto and Parke-Davis peptones with charcoal improves these for spore formation, but Witte and Proteose peptone do not respond. The per cent of sporulating cells can be increased by adsorption of the medium with kaolin, ferric hydroxide, or aluminum hydroxide. Most effective adsorption by charcoal occurs in the pH range of 3–5.

The action of agar and of charcoal in improving Bacto-peptone as a medium for sporulation is dissimilar.

REFERENCES

Brunstetter, B. C., and Magoon, C. A. 1932 Studies on bacterial spores. III. A contribution to the physiology of spore production in *Bacillus mycoides*. J. Bact., **24**: 85–122.

Conklin, M. E. 1934 Mercurochrome as a bacteriological stain. J. Bact., **27**: 30–31.

Williams, O. B. 1931 Bacterial endospore formation in media of varying biologic value. Proc. Soc. Exptl. Biol. Med., **28**: 615–617.

THE TOXIC EFFECT OF SPLENIC EXTRACTS ON STREPTOCOCCUS HEMOLYTICUS

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INTRODUCTION

The problem of Elective Localization has long been a subject of controversy among those who have observed the peculiar tendency of certain bacteria, especially streptococci, to localize and produce lesions in selected organs. In this connection, it has been shown by Rosenow (1914) that as non-virulent streptococci became virulent through successive passage through animals, the site of localization with production of lesions changed markedly. When the virulence was low the selected site of infection was almost wholly avascular tissue; when the virulence was moderate, focal lesions of the kidney were prone to develop along with cholecystitis and ulcers of the stomach; and when the virulence was high, death from bacteremia occurred.

While there is little doubt that this selective action of the streptococcus is due in part to change in virulence, it cannot be considered the sole governing factor. The fact that the organs themselves may offer resistance must also be taken into account. When one considers that streptococci of low virulence attack strictly avascular tissue and those of high virulence, vascular tissue, this relationship between the virulence of infective organisms and the resistance of infected organs seems established.

It is known from the work of Gay and his collaborators (1923, 1924 and 1926) that those tissues which are concerned in immunization, namely, those of the reticuloendothelial system, also offer the greatest resistance to local lesions. This, therefore, might account for the fact that tissues of this system are attacked only by bacterial organisms of the highest virulence.

In our laboratories we have been able to isolate from varied organic sources (Fardon *et al.*, 1937; Sperti *et al.*, 1937; Fardon and Ruddy, 1937; Norris and Kreke, 1937; Loofbourow and Morgan, 1938; Ruddy, 1939), including bacterial organisms themselves (Loofbourow and Morgan, 1940), substances which when allowed to act on certain bacteria greatly stimulated their growth and other metabolic processes. In more recent work, substances of an inhibitory nature, which might be responsible to some degree for an organ's inherent immunity, have also been isolated (Cook *et al.*, 1941; Schroeder and Hollencamp, 1941).

In an attempt to further this latter work, we selected the spleen as the logical organ from which to obtain these inhibitory materials. This selection was made in accordance with the concept of elective localization and the well known fact that infections of the spleen are almost wholly of secondary or hematogenous origin, implying therefore extreme virulence of the infecting agent or extreme resistance of the tissue subjected to the infection. It was thought that the in-

herent immunity or resistance of the organ might be explained to some degree by the presence of inhibitory substances and that these substances might be susceptible to isolation by ordinary chemical methods.

EXPERIMENTAL

Human spleens showing no gross pathology were secured at autopsy from a number of hospitals. These organs were thoroughly minced and alternately frozen and thawed 3 times in an equal volume of Drew's solution (210 ml.). The material was filtered through a Büchner filter and the filtrate was then centrifuged. To 100 ml. of a clear supernatant fluid was added periodically, over an interval of several days, enough 95 per cent alcohol to produce a final alcoholic concentration of 80 per cent. The precipitate which was formed was filtered off and the filtrate concentrated *in vacuo* at 30°C. to a volume of 100 ml. (total solids present 2 g.; pH 7.0). Sterilization of the material was accomplished by passing through a Seitz filter.

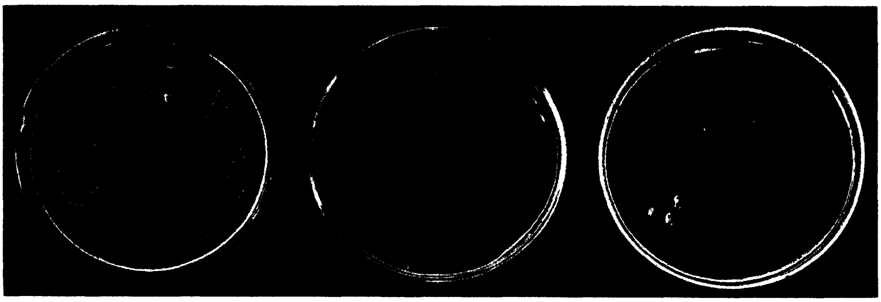

FIG. 1. A. *S. hemolyticus*. Plated from 3-day old culture on brain heart infusion broth (4/1/41)
B. *S hemolyticus*. Plated from 3-day old culture on brain heart infusion broth + 0.5 per cent splen ext. no. 6 (4/1/41)
C. *S. hemolyticus*. Plated from 3-day old culture on brain heart infusion broth + 1 0 per cent spleen ext no 6 (4/1/41).

The following series of experiments were conducted a number of times (6) on hemolytic streptococci obtained from 3 different sources. The results in each instance were similar.

A series of 3 petri plates, each containing 20 ml. of beef-heart infusion agar, was prepared. One served as control, while to the other 2 was added spleen extract in concentrations of 0.5 and 1.0 per cent respectively. (In this and all the other experiments the spleen extract was evaporated to dryness before incorporating it in the media. This was done to eliminate the possible effect of any alcohol which might have been left in the sample after *in vacuo* concentration). Each plate was then inoculated with a 2-day broth culture of hemolytic streptococcus, isolated from a severe mastoid infection, and incubated at 37.5°C. After 1 day the control plate showed normal colonies, while the plates containing the spleen extract showed no growth even after 7 days of incubation. A chart showing the results of these experiments is seen in figure 1.

In order to determine whether the extract was germicidal or merely inhibitory,

a second experiment was performed. A series of 3 tubes, each containing 5 ml. of brain-heart infusion broth, was inoculated with hemolytic streptococcus. Here again the 2 experimental tubes contained 0.5 and 1.0 per cent spleen extract respectively, while the third served as a control. After 2 days' incubation inoculations were made from each tube onto 3 separate petri plates containing plain brain-heart infusion agar. In figure 2 are shown the results of 1 day of incubation of these plates.

FIG. 2. CHART ILLUSTRATING THE EFFECT OF SPLEEN EXTRACT ON THE GROWTH OF THREE STRAINS OF STREPTOCOCCUS HEMOLYTICUS

	EXAMINATION OF PLATES		
	3 days after inoculation	5 days after inoculation	7 days after inoculation
Exp. 1, strep. strain 1			
Control plate	Good growth	Very good growth	Very good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth
Exp. 2, strep. strain 1			
Control plate	Good growth	Very good growth	Very good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth
Exp. 3, strep. strain 2			
Control plate	Good growth	Good growth	Good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth
Exp. 4, strep. strain 2			
Control plate	Good growth	Good growth	Good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth
Exp. 5, strep. strain 3			
Control plate	Good growth	Good growth	Good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth
Exp. 6, strep. strain 3			
Control plate	Good growth	Good growth	Good growth
Exp. plate 1, 0.5% spleen ext.	No growth	No growth	No growth
Exp. plate 2, 1.0% spleen ext.	No growth	No growth	No growth

From this it is quite evident that the extract is definitely germicidal, since mere inhibition in the tubes would have manifested itself by growth when the cultures were transferred to petri plates, as was the case with the plate cultured from the control tube (cf. fig. 3).

In order to determine the lowest concentration of the extract which was effective, a series of tubes containing brain-heart infusion broth and varying concentrations of spleen extract ranging from .05 to 1.0 per cent (.05, 0.1, 0.2, 0.5, 1.0

Fig. 3. Chart Showing Spleen Extract to Be Germicidal and Not Merely Inhibitory for Three Strains of Streptococcus Hemolyticus

	Examination of tubes and plates				
	2 days after tube inoculation		3 days after plate inoculation	5 days after plate inoculation	7 days after plate inoculation
Exp. 1, strep. strain 1		Transferred to control plates			
Control tube..........	Good growth		Good growth	Good growth	Good growth
Exp. tube 1........... 0.5% spleen ext.......	No growth		No growth	No growth	No growth
Exp. tube 2........... 1.0% spleen ext.......	No growth		No growth	No growth	No growth
Exp. 2, strep. strain 2					
Control tube..........	Good growth		Good growth	Good growth	Good growth
Exp. tube 1........... 0.5% spleen ext.......	No growth		No growth	No growth	No growth
Exp. tube 2........... 1.0% spleen ext.......	No growth		No growth	No growth	No growth
Exp. 3, strep. strain 3					
Control tube..........	Good growth		Good growth	Good growth	Good growth
Exp. tube 1........... 0.5% spleen ext.......	No growth		No growth	No growth	No growth
Exp. tube 2........... 1.0% spleen ext.	No growth		No growth	No growth	No growth

Fig. 4. Chart Illustrating Lowest Concentration of Spleen Extract Germicidal for Streptococcus

	Examination of tubes		
	3 days after inoculation	5 days after inoculation	7 days after inoculation
Exp. 1, strep. strain 1			
Control tube....................	Good growth	Very good growth	Very good growth
Exp. tube 1, 1.0% spleen ext....	None	None	None
Exp. tube 2, 0.5% spleen ext....	None	None	None
Exp. tube 3, 0.2% spleen ext....	None	None	None
Exp. tube 4, 0.1% spleen ext....	None	None	None
Exp. tube 5, .05% spleen ext...	Slight growth	Good growth	Good growth
Exp. 2, strep. strain 2			
Control tube....................	Good growth	Very good growth	Very good growth
Exp. tube 1, 1.0% spleen ext....	None	None	None
Exp. tube 2, 0.5% spleen ext....	None	None	None
Exp. tube 3, 0.2% spleen ext....	None	None	None
Exp. tube 4, 0.1% spleen ext....	None	None	None
Exp. tube 5, .05% spleen ext...	Slight growth	Good growth	Good growth
Exp. 3, strep. strain 3			
Control tube....................	Good growth	Very good growth	Very good growth
Exp. tube 1, 1.0% spleen ext....	None	None	None
Exp. tube 2, 0.5% spleen ext....	None	None	None
Exp. tube 3, 0.2% spleen ext....	None	None	None
Exp. tube 4, 0.1% spleen ext....	None	None	None
Exp. tube 5, .05% spleen ext...	Slight growth	Good growth	Good growth

per cent) were prepared. After inoculation and incubation of 7 days, growth occurred only in the control and the tube containing .05 per cent spleen extract (cf. fig. 4). Inoculation from these tubes onto petri plates showed no growth in concentrations as low as 0.1 per cent, thus indicating 0.1 per cent to be the lowest concentration which was germicidal for the organism. In connection with this it is interesting to note that, in an attempt to isolate the active factor from the spleen extract, one-half of the initial 2 g. of solid secured was found to be inactive salt, probably derived from the Drew's solution, so that the 0.1 per cent of solid which proved germicidal in the above experiment, really represented only .05 per cent of active material or 1 part in 2,000.

The preceding experiments were repeated on the 3 strains of hemolytic streptococci using beef spleen extracts prepared in a manner identical to that for the human spleens. Similar results were obtained in every instance. Preliminary experiments on other organisms, including pneumococci, staphylococci, and escherichia coli, seem to indicate that the germicidal effect of both the human and beef extracts is specific for streptococci.

DISCUSSION

From the foregoing work there is no doubt that the spleen contains some factor (or factors) which *in vitro* have a bactericidal effect on hemolytic streptococci. Whether or not this factor is concerned in the specific resistance of the organ is at present difficult to say. It may be stated, however, that from its ability to withstand sterilization, even in acid solution, and its extreme stability to light and air, the substance is of a different nature than the so-called "antibodies" and other defense mechanisms concerned in natural and acquired immunity. The factor appears to be of the nature of a distinct chemical substance, capable of being isolated by chemical procedure. This is in accordance with the findings of other members of our laboratories who have isolated, from other sources, inhibitory and stimulatory factors of a similar nature.

Work is now in progress on the toxicity of this extract and its relative merits *in vivo* on streptococcic infections in animals.

SUMMARY

1. A factor has been obtained from both human and beef spleens, which *in vitro* shows germicidal activity for *Streptococcus hemolyticus* in concentrations as low as 0.1 per cent (1:1,000, 1:2,000).

2. The factor appears to be of the nature of a distinct chemical substance which is resistant to heat and acid.

3. This factor might account, to some degree, for the inherent resistance of the spleen to infection.

REFERENCES

Cook, E. S., Kreke, C. W., Giersch, Sr. M. C., and Schroeder, Sr. M. P. 1941 A growth-depressant substance from yeast. Science, **93**: 616–617.

Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. V. 1937 Stimulating materials obtained from injured and killed cells. Nature, **139**: 589.

Fardon, J. C., and Ruddy, Sr. M. V. 1937 A respiratory-stimulating factor. Studies Inst. Divi Thomae, 1: 41–51.

Gay, F. P. 1923 On local and general immunity. J. Immunol., 8: 1–10.

Gay, F. P. 1924 Local resistance and local immunity to bacteria. Physiol. Revs., 4: 191–214.

Gay, F. P. 1926 Local or tissue immunity. Arch. Path. Lab. Med., 1: 590–604.

Gay, F. P., Clark, A. R., and Linton, R. W. 1926 A histological basis for local resistance and immunity to streptococci. VIII. Studies in streptococcus infection and immunity. Arch. Path. Lab. Med., 1: 857–880.

Gay, F. P., and Morrison, L. F. 1923 Clasmatocytes and resistance to streptococcus infection. V. Studies in streptococcus infection and immunity. J. Infectious Diseases, 33: 338–367.

Loofbourow, J. R., and Morgan, Sr. M. N. 1938 Effect of respiratory and growth-stimulating factors from yeast and malt combings on bacterial growth. Studies Inst. Divi Thomae, 2: 113–127.

Loofbourow, J. R., and Morgan, Sr. M. N. 1940 Investigations of the production of growth-promoting and growth-inhibiting factors by ultraviolet irradiation of micro-organisms. J. Bact., 39: 437–453.

Norris, R. J., and Kreke, C. W. 1937 Three metabolic stimulating factors. Studies Inst. Divi Thomae, 1: 137–162.

Rosenow, E. C. 1914 Transmutations within the streptococcus-pneumococcus group. J. Infectious Diseases, 14: 1–32.

Ruddy, Sr. M. V. 1939 The specific action of two stimulating factors upon the respiration of yeast and liver cells. Arch. exptle. Zellforsch., 22: 599–606.

Schroeder, Sr. M. P., and Hollencamp, C. 1941 Growth factors for bacteria. Studies Inst. Divi Thomae, 3: 193–204.

Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. C. M. 1937 Proliferation-promoting substances from cells injured by ultraviolet radiation. Nature, 140: 643–644.

FACTORS AFFECTING THE BEADING OF THE TUBERCLE BACILLUS STAINED BY THE ZIEHL-NEELSEN TECHNIQUE

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Koch first described the beaded forms of the tubercle bacillus and regarded the beads as evidence of spore formation. Later investigators attempted to correlate the beaded forms with the age and the virulence of the microorganism. These studies, however, have not been fully confirmed.

In the present paper, data will be submitted to show that the beads exhibited by tubercle bacilli stained with carbol fuchsin are greatly influenced by the preparation of the dye used, by the presence of electrolytes in the system, and by subsequent washing with alcohol and other organic solvents.

EFFECT OF ELECTROLYTES

A four-weeks-old culture of virulent tubercle bacilli, Strain H_{37}, grown in Long's synthetic medium, was transferred to a Buchner filter, and the bacilli freed from the medium by washing with distilled water. The washed bacilli were ground in an agate mortar and then suspended in distilled water. Twenty films of tubercle bacilli were prepared by spreading one loopful of this suspension on clean glass slides. The films were air-dried. The solution of carbol fuchsin used in this experiment was prepared as follows: To 10 ml. of a saturated solution of pararosaniline hydrochloride (Lot #9155 from the National Aniline Company, Buffalo, N. Y.) in 95 per cent ethyl alcohol, there were added 10 ml. of alcohol and 180 ml. of 5 per cent aqueous phenol. This solution of carbol fuchsin was divided into two parts:

A. 95 ml. carbol fuchsin + 5 ml. distilled water.

B. 95 ml. carbol fuchsin + 5 ml. 10 per cent NaCl solution.

Ten films were flooded with solution A (no salt) and ten films with solution B. The slides were heated in an electric oven at 90°C. for twenty minutes. The excess dye was poured off, the slides washed with acid alcohol for thirty seconds, and then thoroughly washed with distilled water and air-dried.

On microscopic examination, the preparations stained with solution A (no salt) revealed the bacilli to be solidly stained, with a slight purplish tinge; no beads could be seen in the cellular bodies (fig. 1). On the contrary, the preparations stained with solution B (i.e., in the presence of NaCl in final concentration of 0.5 per cent) showed intense beading. In many instances the beads within the bacilli gave the appearance of a chain of cocci. The beads were of a dark purplish color, whereas the cellular body itself was of a faint pink tinge. Each bacillus exhibited from 1 to 5 beads, irregular in distribution and of a diameter greatly exceeding that of the bacillus itself (fig. 2). This experiment was repeated and confirmed many times.

Similar results were obtained when the following electrolytes were used instead of sodium chloride: sodium acetate, sodium nitrate, sodium sulfate, zinc acetate. Unsatisfactory beading was obtained with calcium chloride, arsenic pentoxide and glucose.

As will be stated later, the addition of NaCl to other samples of basic fuchsin invariably produced an increase in the number of beads.

EFFECT OF WASHING THE STAINED PREPARATIONS WITH ALCOHOL

A number of films of tubercle bacilli were stained as described above using again pararosaniline hydrochloride (Lot #9155, National Aniline Co.) in the presence of NaCl (Solution B, above). Following decolorization with acid alcohol, some of the slides were washed for thirty seconds with 95 per cent ethyl

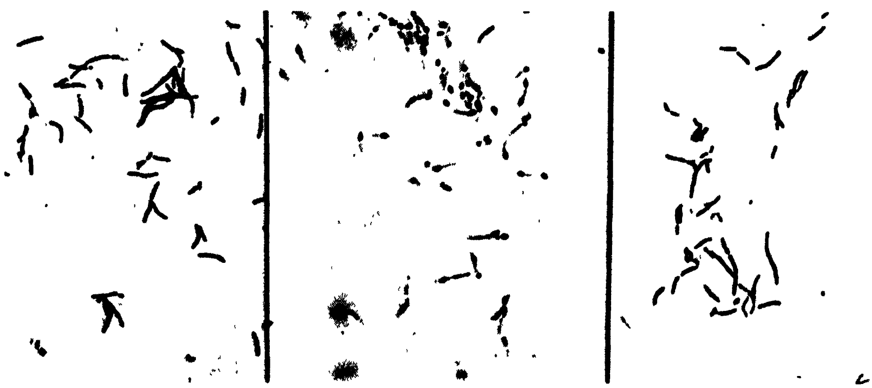

FIG. 1 FIG. 2 FIG. 3

FIG. 1. FILM OF TUBERCLE BACILLI STAINED WITH CARBOL FUSCHIN
No electrolytes added to the staining system The bacilli are solidly stained, no beads

FIG 2. FILM OF TUBERCLE BACILLI STAINED WITH CARBOL FUCHSIN
Electrolytes added to the staining system Bacilli show intense beading

FIG. 3. THE FILM OF BEADED TUBERCLE BACILLI, FIG. 2, WAS WASHED WITH ETHYL ALCOHOL 95 PER CENT
The beads have disappeared and the bacilli are solidly stained

alcohol (no HCl added). All slides were then washed with distilled water and air-dried.

On microscopic examination, the films which had been treated only with acid alcohol, again showed the presence of numerous large purple beads. On the contrary, the preparations which had been washed with neutral alcohol following decolorization with acid alcohol showed bacilli evenly stained, of a pink color. In only two of the ten preparations were beads found and then only in very small numbers (fig. 3).

It has been confirmed with other samples of fuchsin that a final washing with neutral alcohol greatly decreases the number of beads. Methyl alcohol, n-propyl alcohol, and isopropyl alcohol have the same property. The action of acetone is at best very slow and doubtful. Ethyl ether, chloroform, carbon-tetra-

chloride, benzene, toluene, xylol, aniline oil, isobutyl alcohol, amyl alcohol, caprylic alcohol are completely inactive. It may be stated at this time that when stained films are immersed in hot water (95°C.) for three to five minutes, one observes first destruction of the beads, then a more or less complete decolorization of the whole preparation.

The process of destruction of the beads by washing with neutral alcohol can be directly observed in wet preparation under the microscope by the following technique: A film of tubercle bacilli prepared on a thin coverslip is stained in the presence of salt as described above to cause the production of the typical beads. The stained coverslip is inverted over a hollow glass slide, the stained surface downward. The coverslip is sealed with vaseline, care being taken to leave between the coverslip and the well of the hollow slide, an opening large enough to insert a Pasteur pipette. By proper focusing, the stained microorganism and beads can be readily seen with the oil immersion lens. By means of a Pasteur pipette, enough neutral alcohol 95 per cent is then introduced into the well of the hollow slide to come in contact with the stained microorganisms. By keeping the stained microorganisms in focus, during the observation, it can be seen that the beads disappear very rapidly upon contact of the alcohol with the surface area, leaving the microorganisms of a pink color and evenly stained. Not infrequently the destruction of the beads is delayed, affording an opportunity to observe more closely the manner in which they disappear. The beads first elongate, then change in color from purple to pink, and finally merge with the body of the bacillus, which then assumes its natural rod-shape form and becomes solidly stained.

The formation of the beads likewise may be observed. A film of tubercle bacilli on a coverslip is stained with carbol fuchsin and sealed as described above. The stained preparation is flooded with acid alcohol until the color of the microorganism changes from red to purplish blue, approximately thirty seconds. The acid alcohol is then removed and replaced with distilled water. The water is repeatedly changed in order to remove the acid alcohol from the well completely. After the bacilli are in contact with the water for about two minutes, the beads appear, but so suddenly that it is not possible to follow their development. The body of the bacillus usually assumes a light pink color when the beads are formed.

EFFECT OF DIFFERENT SAMPLES OF BASIC FUCHSIN

For the sake of clarity, only experiments performed with one single sample of dye have been described so far. In fact, the production of beads in films of tubercle bacilli stained by the Ziehl-Neelsen technique is greatly influenced by the sample of the dye used. Some dyes cause beading in the absence of electrolytes. With others, the addition of the electrolyte is necessary to produce the effect. Whenever they occur, washing with neutral alcohol at the end of the staining process results in the disappearance of the beads, or a great reduction in their number.

It may be of some interest to summarize briefly the properties of some of the

TABLE 1

The effect of the preparation of basic fuchsin, of the dilution of the dye, and of sodium chloride on the beading of tubercle bacilli

DYE USED	SOURCE	SOLUBILITY IN 95 PER CENT ALCOHOL	ASH	FINAL CONCENTRATION OF DYE	BEADING	
					Without NaCl	With NaCl
		per cent	*per cent*	*per cent*		
Pararosaniline acetate, lot #8772	National aniline	13.0	1.99	.3 .14	Few beads No beads	100% 95%
Pararosaniline acetate, no lot number	Coleman and Bell	7.42	.16	.5 .3 .14	50% 20–30% No beads	100% 100% 50%
Pararosaniline acetate, lot #6820	National aniline	11.42	.03	.5 .3 .14	50% Few beads No beads	100% 80% 50%, beads small
Pararosaniline hydrochloride, no lot number	Coleman and Bell	5.06	.4	.5 .3	No beads No beads	Many beads Many beads
Pararosaniline sulphate, lot 3211	National aniline	1.4	0.0	.14	No beads	Few beads
Basic fuchsin NF 31, pararosaniline acetate	National aniline	9.45	.93	.5 .3 .15 .075	Many beads Many beads Nearly 100% but not large or distinct 5% beads but not large or distinct	Many beads Many beads Many beads Few beads
Basic fuchsin CE 16, rosaniline hydrochloride	Coleman and Bell	5.21	.02	.5 .3 .15	No beads No beads No beads, faintly stained	Few beads Few beads (?) No beads, better stain
Basic fuchsin, rosaniline hydrochloride, lot #391205	Coleman and Bell	6.07	.01	.5 .3 .15 .075	60–70% beads No beads No beads No beads	100% 90% 50%, indistinct 10–20%
Pararosaniline hydrochloride, lot #9155	National aniline	6.87	1.17	.3 .14	No beads No beads	100% Very few beads

dyes which we have studied. It is noteworthy that solutions of the acetate and hydrochlorides of pararosaniline exhibit some striking physical differences;

the acetates appear as pinkish, opaque colloidal suspensions, whereas the hydrochlorides are transparent, of a deeper purple color. Addition of salt to the hydrochloride solution renders them opaque and pink, like the acetates.

EFFECT OF DILUTION OF DYES

As will be seen from the results presented in table 1, some of the dyes which failed to give beads when used in the usual concentration, in the absence of NaCl, did give some beads in the absence of salt when the dye concentration was greatly increased. Conversely, it has been found that the ability of any dye to produce beads in the presence of NaCl is much decreased when the dye concentration is reduced. This is illustrated in the following experiment: The pararosaniline hydrochloride Lot #9155 of the National Aniline Company, when made in 0.3 per cent dilution, gives a slightly opaque solution on addition of 5 per cent aqueous phenol. When used in the Ziehl-Neelsen technique on tubercle bacilli, Strain H_{37}, this dye solution does not produce any beads in the absence of NaCl, whereas all bacilli are beaded when 0.5 per cent NaCl is added to the staining mixture. When used in concentration of 0.14 per cent, the same dye gives a clear solution on addition of phenol. Tubercle bacilli stained with this solution show only a few scattered beads even in the presence of NaCl.

A concentration of 0.3 per cent of the pararosaniline acetate (Lot #8772, National Aniline Co.) gives a very opaque suspension on addition of phenol. Under these conditions, this dye gives a few beads even in the absence of NaCl, and marked beading of all bacilli in the presence of the salt. When the same dye is used in a final concentration of 0.14 per cent, a slightly opaque solution is obtained on addition of phenol. This more dilute preparation gives no beads at all in the absence of NaCl, and only partial beading of the bacilli in the presence of the salt.

As will be seen in table 1, the pararosaniline sulfate (Lot #3211, National Aniline Co.) is only poorly soluble in alcohol, and when used in final concentration of 0.14 per cent gives only very few beads even in the presence of salt.

DISCUSSION

The observations described in the present paper indicate that the presence of beads in films of tubercle bacilli stained by the Ziehl-Neelsen technique depends not only upon the existence of structures of the bacterial cell, but to a large extent upon the conditions under which the staining reaction is carried out. Films prepared from the same suspension of tubercle bacilli and stained under the same conditions, may present uniformly, solidly-stained bacilli, or bacilli exhibiting beads in varying number, depending on the preparation of carbol fuchsin used. Addition of small amounts of electrolytes to the dye solution greatly increases the number of beads with all dyes, even with those which never produce any beads in the absence of salt. In preparations stained in the presence of salt, the bacilli often appear as chains of heavily-stained purple bodies, larger than the diameter of the cell proper, whereas the body is stained only faintly pale pink. The production of beads is much decreased even in the presence of salt, when the dyes are used in lower concentrations than is the usual practice.

In all cases, washing of the stained preparations with neutral alcohol (following decolorization with acid alcohol) removes practically all the beads and leaves most of the bacillary bodies as evenly-stained rods of a pink tinge. The production of beads by the addition of electrolytes to the staining system, and their destruction with neutral alcohol can be seen directly under the microscope when stained preparations are treated with these reagents on a coverslip using a hollow slide, and this holds true for cultures of virulent and avirulent tubercle bacilli, and for tubercle bacilli present in sputum, pleural fluid, etc. The addition of electrolytes to the solution of carbol fuchsin causes a precipitate, which, when separated by centrifugation, is readily soluble in alcohol; these observations suggest that the bead formation observed in the presence of salt, or even without salt in the case of some samples of dyes, may be essentially nothing but a precipitation of the dye on the surface of, or within the bacterial cell. Whether definite cellular structures participate in the phenomenon by providing a nucleus which facilitates the reaction, or whether the phenomenon is only a physical artifact, cannot be decided at the present time. It appears justified, however, to suggest that before any attempt can be made to correlate the staining character of the tubercle bacillus with its biological properties (age, virulence, etc.) it is essential to gain a better understanding of the many factors which affect the result of the staining process. In addition to the type of beads described in the present study, there are, of course, the tiny granules seen in tubercle bacilli stained by the Ziehl-Neelsen technique, particularly noticeable in old cultures. The granules, unlike the typical beads, do not disappear when washed with neutral alcohol, and appear, therefore, to be of a different nature.

SUMMARY

The production of beads in films of acid-fast microorganisms stained by the Ziehl-Neelsen technique is greatly influenced by the preparation of basic fuchsin used in the staining process, and by the concentration of the dye solution.

With all preparations of fuchsin, addition of electrolytes greatly increases the number of beads. Washing of the stained preparation with neutral alcohol, following the decolorization with acid alcohol, destroys all, or a large majority of the beads.

The effect of electrolytes and of alcohol on the production and destruction of the beads can be directly followed under the microscope in wet preparations.

We wish to acknowledge our thanks to Dr. René J. Dubos of the Rockefeller Institute for many helpful suggestions.

FURTHER STUDIES ON PROTEUS HYDROPHILUS, THE ETIOLOGICAL AGENT IN "RED LEG" DISEASE OF FROGS

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INTRODUCTION AND HISTORICAL REVIEW

The frog has long been used as an experimental subject in various fields of research, yet rarely have reports been published concerning diseases to which it is naturally susceptible.

The first investigations on septicemic diseases of frogs were apparently those of Ernst (1890), who reported the isolation of a bacterium from the blood of infected frogs.

A year later Sanarelli (1891) described an organism isolated from frog's blood. He named it *Bacillus hydrophilus fuscus.* He did not believe it to be identical with that reported by Ernst. Sanarelli found that *Bacillus hydrophilus fuscus* was pathogenic for such cold-blooded animals as the frog, toad, salamander, lizard, sunfish and the fresh water eel. He also observed that injection of this organism into warm-blooded animals (guinea pig, rabbit, dog, cat, mouse, chicken and pigeon) resulted in death within a relatively short time. He described the growth of the bacillus on plain agar, glycerol agar, gelatin, blood serum and potato, and suggested that its natural habitat is water, since he found it to be present in two of twenty-six water supplies examined by him.

In 1893 Trambusti isolated what proved to be the same species from diseased frogs in his laboratory. His investigations of *Bacillus hydrophilus* were primarily concerned with the isolation of certain toxic metabolic products produced by it. He concluded from his experiments that the toxic products were of two kinds, one soluble and the other insoluble in alcohol. He made an attempt to determine the physiological action of both of these agents, and also of pure cultures of the organism, upon experimental animals.

An epizootic disease among frogs was described in 1893 by Roger. He found that the viscera and blood of these amphibia contained a small bacillus in pure culture. The same organism was easily obtained from the water of the aquarium which housed the infected frogs. From these observations this worker concluded that the organism was the same as that which Sanarelli had observed in a similar epizootic. A brief description was given by Roger of the gross pathological findings, in which he emphasized the appearance of general hemorrhagic areas in all species of artificially infected animals.

The first adequate description of the morphological, cultural, biochemical and

[1] The material published in this paper was taken from the thesis presented by the junior author in partial fulfillment of the requirements for the Master of Science degree at the University of Connecticut. For brevity, considerable detail has been omitted. For further detail, readers are referred to the thesis in the University of Connecticut library.

pathogenic properties of *Bacillus hydrophilus* was published by Russell in 1898, and his findings confirmed much of the work of Sanarelli and Trambusti. He made an extensive study of the gross pathological and histopathological findings of diseased frog tissues. His investigations on the metabolic products derived from the microorganisms showed that they elaborated two potent toxins, as was previously reported by Trambusti. One of these toxins resembled digitalis in its action, and the other veratrin. Russell concluded that no definite statement could be made concerning the route of infection in frogs. He thought, however, that infectious material was brought to the laboratory with the frogs and that infection became established through superficial skin lesions.

The common name of this frog disease in this country and in Canada is "red-leg." This designation first appeared in an article by Emerson and Norris in 1905. They claimed that the name is ideally suited, since it attracts attention to the most outstanding pathognomonic lesion common to diseased frogs, namely petechial haemorrhages on the surface of the abdomen and legs, varying from a light red to a deep scarlet color. Although these investigators observed some variation from the morphology as described by previous workers, they identified *Bacillus hydrophilus fuscus* of Sanarelli as the essential etiological agent. In the course of their studies they made several original observations both upon the pathological state of frogs and upon the products of the bacterial cultures. A series of carefully controlled experiments demonstrated that, while temperatures slightly above freezing have no harmful effect upon frogs, such temperatures completely control all manifestations of the disease in inoculated or naturally diseased frogs. They also found that the severity of the infection was definitely correlated with the destruction of the erythrocytes of the diseased frogs. This they believed was due to the action of the microorganism directly. Emerson and Norris concluded that the disease had a wide distribution throughout North American and Europe.

The substitution of the name *Proteus* for *Bacillus* originated with Weldin and Levine (1923). Bergey (1939) recognized the etiological entity of "red-leg" as *Proteus hydrophilus*. Throughout the remainder of this investigation, therefore, the species will be referred to as *Proteus hydrophilus*.

GROSS PATHOLOGY OF "RED-LEG" DISEASE IN FROGS

The information presented here on the gross pathology of "red-leg" disease in frogs was secured by observing over a hundred spontaneously infected animals and an additional thirty which were artificially infected with pure cultures of *Proteus hydrophilus*.

The malady which resulted from either spontaneous or artificial infection of frogs with *P. hydrophilus* was characterized at the onset by a distinct sluggishness of the diseased animal. Within one or two days hemorrhagic areas more or less uniform in appearance were observed on the ventral surface of the body. An extensive edema of the abdomen and thighs occurred several hours before the frogs succumbed to the disease.

Post-mortem examinations consistently revealed the presence of considerable edematous fluid beneath the skin of the abdomen and thighs. Small hemorrhagic

areas were occasionally seen on the surface of the tongue accompanied by a slight amount of blood-tinged exudate in the mouth. Multiple petechial hemorrhages were always observed on the surface of the abdominal and thigh musculatures. In a few instances small ulcers penetrated from the abdominal cutaneous tissue to the rectus abdominal musculature. The peritoneal cavity of medium-sized infected frogs contained two or three milliliters of a hemolyzed bloody exudate in which were found large numbers of the infecting organisms.

The heart muscle was always pale and flaccid. In many of the infected frogs the lungs appeared highly congested, while in others no abnormal changes were noticed. A parasite, which is probably *Distonum cylindraeceum*, was obtained from the lungs of approximately one-fourth of the frogs examined.

The blood vessels on the surface of the stomach and intestine were intensely congested, while the organs themselves appeared greatly distended. An extremely viscid, bloody material exuded from both the stomach and the intestine when they were severed. The fluid of the gall bladder varied from a light yellow to a deep green color. It is of interest to note that *Proteus hydrophilus* was easily isolated in relatively large numbers from the yellow fluid, but rarely from the deep green fluid of the gall bladder. The liver appeared dark brown and mottled. The spleen seemed considerably enlarged and roughened due to the presence of abnormal uniform protusions of the spleenic capsule. No abnormal changes were observed in the kidneys.

Proteus hydrophilus was readily recovered from the various organs and the exudate and blood of infected frogs. Many of the data obtained were in agreement with the findings of Russell (1898) and Emerson and Norris (1905). The last two workers observed that a large number of infected frogs which presented only small areas of congestion and few vesicles recovered within a few weeks after the onset of the infection. In the present investigation recovery of frogs from "red-leg" disease was never observed after the clinical symptoms were once apparent.

In several instances *Proteus hydrophilus* was obtained in pure culture from the heart blood of dead frogs in which no clinical manifestations or gross pathological changes were observed. If we assume that the death of the amphibia was caused by invasion of *Proteus hydrophilus*, the absence of any apparent lesions may be explained on the basis of extreme susceptibility on the part of the host. Topley and Wilson (1936) state that when the host possesses no immunity to the etiological entity, it will succumb to a bacteremic infection in which local lesions are rarely observed.

METHOD OF ISOLATING PROTEUS HYDROPHILUS AND HISTORY OF CULTURES

Blood broth and blood agar having the following basic composition were the mediums used in isolating the organism:

	per cent
Bacto peptone	1.0
Savita (yeast concentrate)	1.0
NaCl	0.5
Distilled water	

Agar (1.8 per cent) was added to the above, in the preparation of a solid medium. The addition of three per cent by volume of sterile defibrinated blood completed the formula.

For primary isolation, a loopful of material (usually heart's blood) was transferred to a tube of blood broth. A second loopful was streaked over the surface of a blood agar plate. Usually, there was good colonial growth on the blood agar plate after from 24 to 48 hours' incubation. Occasionally, no colonies developed on the agar but there was growth in the blood broth. In such cases streak plates from these broth cultures yielded good growth of the organism. All broth and agar plate cultures for primary isolation were incubated at 25°C.

One hundred and twenty-one strains were isolated from 96 sources, most of these being infected frogs. The discrepancy between the total number of isolations and the sources can be explained by stating that in several instances two isolations were made from the same animal, one from the heart blood and one from the gall bladder. Eight strains were secured from dead frogs which showed no gross pathological evidence of "red-leg" disease. Ten isolations were made from aquarium water in which infected frogs were kept. Eighteen came from either the heart's blood or gall bladder of apparently healthy frogs. Three strains were obtained from the water of an aquarium containing frogs which appeared healthy. After assurance that the isolations were pure, they were grown and stored on blood agar slants.

CELL MORPHOLOGY AND GROWTH STUDIES

Primary isolations of *Proteus hydrophilus* may possess as many morphological variations as the species has had names in the past. In smears made from the heart's blood of infected frogs and stained by Wright's method (Gradwohl, 1938) the organism presented a wide variety of shapes. Plump, short, encapsulated diplobacilli measuring approximately 2.0 microns in length and about 0.8 micron in width predominated. The ends of many cells were rounded, while others were truncate or pointed. Bipolar staining was usually observed in the cells having rounded ends. Clavate and elongated dumb-bell forms were present to a considerable extent in the smears. Filamentous variants were never observed. Encapsulated coccobacilli and lance-shaped varieties of the organism were fairly common. Narrow elongated granular forms were present in very small numbers. These observations were made on heart's blood from which *Proteus hydrophilus* was obtained in pure culture.

When grown on standard nutrient agar for a period of 18 hours at 30°C. and stained by Gram's method, *Proteus hydrophilus* appeared as a gram-negative straight rod with rounded ends and measured about 2.5 microns by 0.6 micron. Under these conditions the organism occurred either singly or in pairs. Irregular forms such as coccobacilli and clavate types were occasionally seen. Bi-polar staining was a very characteristic feature of newly isolated strains. The organism was non-sporing. It possessed a capsule and a single flagellum which was readily demonstrable by Liefson's method of staining (1930) (Figure 1). Bergey (1939) states that this organism has peritrichous flagella, but Russell

(1898), on the other hand, reported monotrichous flagellation. In the present study no non-motile strains of *Proteus hydrophilus* were observed following their growth in nutrient broth at 30°C. for 18 hours.

Two strains, P_2 and B-f-1, were selected as representative of the entire group of isolations and were subjected to further study. These two strains differed from each other only in their serological relationships. When grown on standard nutrient agar plates for 24 hours at 37°C., the translucent colonies which formed were approximately 2 mm. in diameter and were creamy white in color. They were slightly convex and circular in appearance, with an entire edge. The internal structure was finely granular and the surface smooth and moist. Growth was butyrous in consistency. The swarming phenomenon described by Cantu (1911) and Moltke (1927, 1929) as being characteristic for members of the

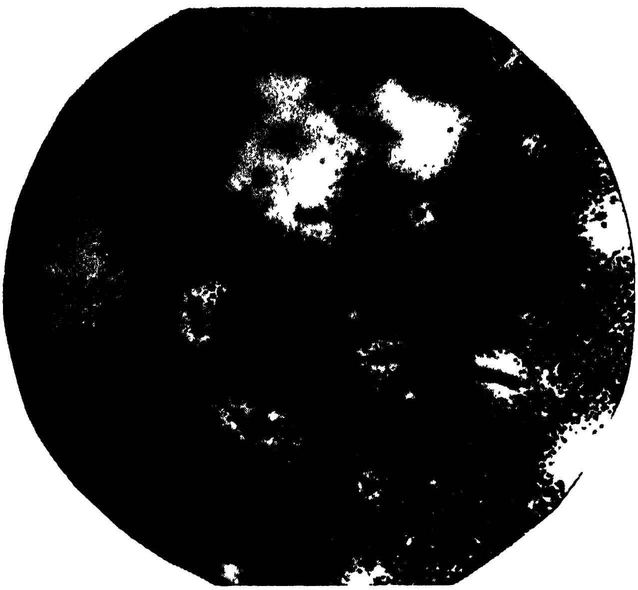

FIG. 1 FLAGELLA OF PROTEUS HYDROPHILUS

Proteus genus was never observed when *Proteus hydrophilus* was cultured on moist standard nutrient agar plates or on any other solid medium.

When grown on 3 per cent ox blood Savita agar plates which were incubated at 37°C. for 24 hours, the two strains produced circular colonies approximately 3 mm. in diameter, surrounded by a zone of beta type hemolysis which was about 1½ mm. in diameter. The colonies were convex, finely granular, smooth, moist and glistening, with an entire edge. Although they seemed to be creamy white in color, a slight brownish tinge was observed in the peripheral portion. This effect may have been produced initially by the red color of the medium; in older cultures, however, it was observed to be a characteristic property of the colony. Distinct central and peripheral areas were seen in all colonies.

Growth on agar slants occurred readily at 30°C. Following an incubation

period of 48 hours, the creamy white growth was abundant, slightly spreading and possessed an undulate edge. The surface was smooth, moist and raised. Old agar slant cultures occasionally became yellowish in color.

Standard nutrient broth was a very satisfactory medium for growth of the organism. Multiplication in this medium was very rapid during a 24-hour incubation period at either 30°C. or 37°C. A dense uniform turbidity was always seen in the cultures examined and a deposit was usually present which broke up completely when the tube was shaken. Occasionally surface pellicles were observed in young cultures. Old broth cultures possessed an offensive odor.

BIOCHEMICAL STUDIES

With only slight differences here and there, the various strains had the following biochemical properties.

Acid and gas were produced from glucose, fructose, galactose, maltose, sucrose, salicin and starch. Raffinose, sorbitol and xylose were not attacked, and action on lactose was, at best, weak and should be described as doubtful. Napiform liquefaction occurred in gelatin tubes. Indole was produced and H_2S also was formed. All strains were Voges-Proskauer positive and all failed to grow in Koser's citrate broth. Methylene-blue milk was reduced and a rennet curd without acid production was formed, followed by peptonization of this curd. Ox, sheep, rabbit, horse and fowl erythrocytes were hemolyzed. Urea was not attacked, in strong contrast to several strains of *Proteus vulgaris* which were urea-positive.

Moltke (1927) stated that two very important properties which distinguish the *Proteus* group from other gram-negative, gelatin-liquefying, rod-shaped bacteria are the production of H_2S and the decomposition of urea. Wolf (1918-19) isolated three members of the *Proteus* genus from three different infections in man and found that they hydrolyzed urea with the formation of ammonia. Approximately 43 per cent of the total nitrogen present in the given medium (urine or urine-containing medium) was transformed into ammonia. Taylor (1928) observed that urine contaminated with *Proteus* organisms possessed an offensive odor which eventually became ammoniacal. He concluded that sterile normal urine, when inoculated with *Proteus* strains from various sources (suppurative wounds, feces and urine), soon became ammoniacal and deposited phosphates. Minning and Ritter (1937) also reported that urea is hydrolyzed with ammonium production by members of the *Proteus* genus.

The comparative action on urea of *Proteus vulgaris* and *Proteus mirabilis* (which are probably one and the same organism) and *Proteus hydrophilus* was studied.

The first medium employed was that of Murray (1916), but none of our cultures would grow in it. Next, a medium of the following composition was used:

	per cent
NaCl	0.5
K_2HPO_4	0.2
$MgSO_4$	0.5
Glucose	1.0
Urea	1.0
Distilled water	

This medium was sterilized by filtration. No growth occurred in it, but when meat extract in a concentration of 0.05 per cent was added all of the organisms grew. With the exception of *Proteus hydrophilus*, all of the cultures produced ammonia. To determine whether meat extract served as a source of ammonia, it was substituted in the original formula for the urea. All species grew in it, but no ammonia was produced by any of them. It was concluded that the *Proteus* species other than *Proteus hydrophilus* do decompose urea.

A few strains and also filtrates from broth cultures of some strains produced a startling zonation effect (Figure 2). This occurred in blood agar made with sheep, fowl, rabbit, horse and ox blood, but the greatest degree of zonation was noted in agar to which ox erythrocytes had been added.

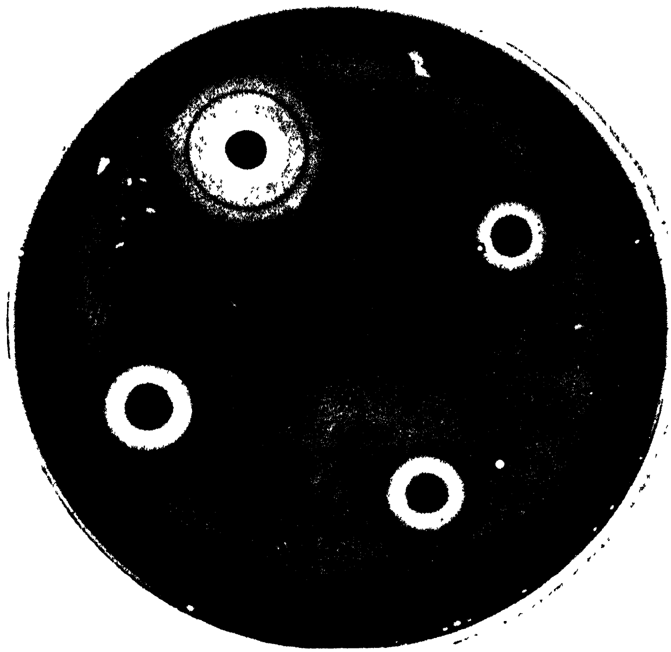

FIG. 2. ZONATION EFFECT OF PROTEUS HYDROPHILUS ON BLOOD AGAR

Brown (1919), Kortenhaus (1929), Rhodes (1938) and others have reported this zonation effect, called "Leisegang ring formation," in blood-agar cultures of streptococci, staphylococci and pneumococci. This phenomenon has been ascribed to a combination of chemical and physical factors.

SEROLOGICAL STUDIES

Antisera were prepared for several strains of *Proteus hydrophilus*. A study of these sera indicated that there were two distinct serological types of this organism in our collection, represented here by the P_2 and B-f-1 strains. It is interesting to note that of 24 normal rabbit sera examined, seventeen showed somatic agglutinins specific for B-f-1, while none reacted with P_2. It is known that normal

rabbits often harbor *Pasteurella cuniculicida* (*Bacillus leptisepticus*) in their nasal passages de Kruif (1922), Webster (1924). This organism is associated with a disease in rabbits called "snuffles." It contains Forssman's antigen (Zinsser *et al*, 1939). On this basis it is possible to explain the presence of so-called "normal" agglutinins found in the serum of rabbits which are specific for the B-f-1 strain of *Proteus hydrophilus*. Only rabbits showing no agglutinins for either strain were used in the preparation of specific *P. hydrophilus* antisera.

Using the above antisera, an attempt was made to determine the relationships of the different isolations to the above two strains. Both flagellar and somatic factors of the cell were considered in this study. It was found that, contrary to the observations of other workers, there was little difference between the flagellar and the somatic titers. The fact that this species is monotrichous may help to explain this finding.

The following conclusions are offered for this phase of the investigation:

1. Two distinct serological types of *Proteus hydrophilus* predominate.
2. Evidence points to the presence of other types of this organism not employed in this study.
3. More than one serological type of the pathogen may be isolated from the body of an infected frog.
4. Strains of *Proteus hydrophilus* vary in the degree of their antigenic relationship to the B-f-1 and P_2 serological types, on the basis of flagellar and somatic agglutination reactions.
5. Strains of the frog pathogen may possess common somatic, but dissimilar flagellar, antigenic components for the serological type B-f-1 strain.
6. The homologous flagellar titers of strains of B-f-1 and P_2 do not exceed the somatic titers of these organisms.

HABITAT

Bergey (1939) states that the habitat of *Proteus hydrophilus* is unknown. Sanarelli (1891) thought that the organism was a normal inhabitant of water, but he was able to isolate it from this source only twice in 26 attempts.

In the present research the waters of seven aquaria used to house apparently normal frogs were examined and *Proteus hydrophilus* was isolated from three of these sources. Proceeding on the hypothesis that in this instance, as in many other infectious diseases, the healthy carrier may be the source of the organism, several frogs from each of the above aquaria were autopsied. The internal organs appeared to be normal, and *Proteus hydrophilus* was isolated from the gall bladder of only three frogs. In these three cases, however, the fluid in the gall bladder was yellow in color, in contrast to the green-colored bile generally found in normal frogs. A fourth isolation was made from the intestine of another frog, but in no other instance was this organism recovered from animals which appeared healthy.

It was easy, as a rule, to secure *Proteus hydrophilus* from the waters of aquaria which harbored infected frogs. However, in a few instances frogs that were segregated in small individual aquaria did not eliminate the organism, even

though pure cultures were secured from the gall bladder of these animals at autopsy. This is in agreement with the findings of Amoss (1922), Topley (1926) and Knorr (1926) in their study of laboratory rodents which were experimentally infected with members of the *Salmonella* group. They concluded that these organisms may be eliminated continuously or intermittently in the feces or urine, being harbored in these instances in the spleen, liver, gall bladder and lymph nodes. Meyer, Neilson and Feusier (1921) claimed that in rodents surviving *Salmonella* infections the persistent carrier state resulted from a bacterial embolism in the gall bladder wall.

The portal of entry necessary for *Proteus hydrophilus* to become established as an infectious organism in the frog appeared to be the abraded skin. Frogs experimentally scratched to break the skin and placed in water containing the organisms developed the typical disease very quickly. Attempts to produce the infection by feeding were unsuccessful.

TAXONOMY

Although the organism causing "red-legs" has been named differently by past investigators as *Bacillus ranicida* (Ernst, 1890; Weldin, 1926–7), *Bacillus hydrophilus fuscus* (Sanarelli, 1891), *Bacterium hydrophilus fuscus* (Chester, 1897), *Bacillus hydrophilus* (Chester, 1901) and *Bacterium ranicida* (Lehman and Neumann, 1931), it is now known as *Proteus hydrophilus*.

Bergey's Manual (1939) recognizes the *Proteus* genus on the following basis:

> Highly pleomorphic rods. Filamentous and curved rods common as well as involution forms. Gram negative. Generally actively motile, possessing peritrichous flagella. Characteristically produce amoeboid colonies, etc., on moist media and decompose proteins. Ferment dextrose and generally sucrose, but not lactose. Do not usually yield a positive Voges-Proskauer test. Urea decomposed.

Topley and Wilson (1936) agree with the above description of this genus and add that mannitol is fermented.

Members of the *Proteus* genus cause coagulation and digestion of casein in milk, and indole may or may not be formed, according to the Medical Research Council (1929). Fifty-three strains of the *Proteus* group were examined by Taylor (1928) who found only three which produced indole. Moltke (1927) reported only 36 strains out of 194 examined as giving the reaction.

As previously stated (Moltke, 1927; Wolf, 1918–9), urea decomposition and the production of hydrogen sulfide are claimed as important characteristics, distinguishing species of the *Proteus* genus from other gram-negative, gelatin-liquefying bacteria.

Proteus hydrophilus, according to the present investigation, has the following properties which are characteristic of the genus: it is gram-negative, actively motile, gives rise to involution forms (present only in the animal body), ferments glucose and sucrose, and produces hydrogen sulfide. Its variable properties include indole and acetyl-methyl-carbinol production and gelatin liquefaction. The absence of the "swarming" phenomenon, urea decomposition,

peritrichous flagella, filamentous and curved rod formation makes it differ from characteristics usually considered significant for the *Proteus* genus.

TOXIN STUDIES

Proteus hydrophilus and bacteria-free culture filtrates of this organism are strongly hemolytic.

An attempt was made to determine whether a hemotoxin was the lethal agent in mice dying from infection with *Proteus hydrophilus*. Erythrocyte counts were made on a series of eight adult mice. These mice were then inoculated with a lethal dose of *Proteus hydrophilus*. They were kept under close observation, and immediately after death the erythrocyte count was repeated. No deviation was found from the original average of 8,000,000 per cu. mm. which would seem to indicate that the mice did not die because of erythrocyte destruction. This parallels the observations of Zinsser, Enders and Fothergill (1939) regarding streptolysin and its relationship to streptococcal infections. They reported that hemolysin has little significance in the lethal effect of streptococci which are strongly hemolytic *in vitro*.

A substance which was definitely toxic for mice, rabbits and guinea pigs was demonstrable in bacteria-free filtrates of *Proteus hydrophilus* broth cultures which were from six to thirty days old. Mice were more resistant to intraperitoneal than to intravenous injection of this material. Heating such a filtrate to the boiling point for one hour reduced the toxicity for mice to about 10 per cent of the original. This substance was quite stable when kept at 10°C. in the dark. Rabbits were given a series of sub-lethal intravenous injections of this filtrate, but the resulting antisera had no protective action for mice subsequently injected with the above filtrate. In contrast, mice immunized with *Proteus hydrophilus* bacterins were not killed by intravenous injection of this toxic substance. These experiments seem to indicate that the toxic material in old broth cultures of *Proteus hydrophilus* is endotoxic in nature.

IMMUNOLOGICAL STUDIES

Kulp, Lackman and Borden (1940) were able to immunize mice and leopard frogs against *Proteus hydrophilus* infection. They employed as immunizing antigen heat-killed suspensions of *Proteus hydrophilus;* also living and heat-killed suspensions of *Proteus mirabilis*. Successful immunization was secured in from 60 to 100 per cent of the animals tested. They were unable to determine any antigenic relationship between the immunizing strains of *Proteus vulgaris* and *Proteus mirabilis*, on the one hand, and the pathogen, *Proteus hydrophilus*, on the other.

This work was repeated and extended in the present investigation. Immunization experiments were conducted with the two serological types of *Proteus hydrophilus*, B-f-1 and P_2. It was shown that a bacterin of one of these types protects frogs against both strains. Antibodies could not be demonstrated in the blood of immunized frogs except in one instance where agglutinins were found in the blood of a large bull-frog which received repeated injections

of bacterin. In the experiments with mice the bacterin was prepared by alternate freezing and thawing of the suspensions until they were sterile. Cross-immunization was secured in mice with B-f-1 and P_2 bacterins prepared in this manner. Blood from mice immunized against one strain did not show the presence of either somatic or flagellar agglutinins specific for the other strain. Antiserum from mice immunized against P_2 bacterin did not confer passive immunity against B-f-1 and *vice-versa*, nor did antisera prepared in rabbits against one of the above types protect mice passively against the other strain of this organism.

An attempt was made to explain the mechanism of immunity in mice. A definite increase in leucocytes was noted in these animals, but since the study of the bactericidal power of the blood was made on the whole blood, the immunity may not be entirely leucocytic. Borden (1936) found that blood from mice which had been immunized with living cultures of *Proteus vulgaris* and *Proteus mirabilis* was bactericidal for *Proteus hydrophilus in vitro*. It is quite possible that both humoral and cellular responses may be involved. This phase of the problem will be studied further.

SUMMARY AND CONCLUSIONS

"Red-leg" disease, an epizootic of aquarium frogs, is a form of septicemia that is caused by an organism which is a member of, or so closely related to, the *Proteus* group as to merit the name *Proteus hydrophilus*.

Proteus hydrophilus is a gram-negative monotrichous rod; it is a more or less pleomorphic, non-sporulating, strongly fermentative, gelatin-liquefying organism. This species produces acetyl-methyl-carbinol, is indole-positive, grows readily on the ordinary nutrient mediums, but does not produce the "swarming" type of growth generally attributed to the *Proteus* genus. It develops a potent lysin for the erythrocytes of several animal species. Certain strains produce a zonation effect on blood agar mediums. A toxic substance has been demonstrated in culture filtrates, which appears to be endotoxic in nature.

There are at least two definite serological types in these species. When employed antigenically as bacterins, each brings about immunity in experimental animals against both types, whereas passive immunization has been demonstrated only against the homologous type.

Frogs which appear normal seem to act as carriers, the infective agent in all probability being carried in the gall bladder.

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REFERENCES

AMOSS, H. L. 1922 Experimental epidemiology. II. Effect of the addition of healthy mice to a population suffering from mouse typhoid. J. Exptl. Med., **36**, 45–69.

BERGEY, D. H., ET AL. 1939 Manual of Determinative Bacteriology. The Williams & Wilkins Company, Baltimore, Md.

BORDEN, A. G. 1936 The effect of a non-specific vaccine on the immunity of mice to pneumococcus type I and *Proteus hydrophilus*. A thesis in the Library of the University of Connecticut.

BROWN, J. H. 1919 Monograph of the Rockefeller Institute for Medical Research, No. 9.

CANTU, C. 1911 Le Bacillus Proteus sa distribution la nature. Ann. inst. Pasteur, **25**, 852–864.

CHESTER, F. D. 1897 A preliminary arrangement of the species of the genus *Bacterium*. Contributions to determinative bacteriology. Part I. Ninth Ann. Rept. Del. College Agr. Expt. Sta., 92.

CHESTER, F. D. 1901 Manual of Determinative Bacteriology. 235.

EMERSON, H., AND NORRIS, C. 1905 "Red-Leg"—an infectious disease of frogs. J. Exptl. Med., **7**, 32–58.

ERNST, P. 1890 Die Frühjahrsseuche der Frösche und ihre Abhängigkeit von Temperature-einflüssen. Beitr. path. Anat., **8**, 203–220.

GRADWOHL, R. B. H. 1938 Clinical Laboratory Methods and Diagnosis. The C. V. Mosby Company, St. Louis, Mo. (2d ed.) 328 and 785.

KNORR, M. 1926 Akute Gastroenteritis und Typhöser Paratyphus. Zentr. Bakt. Parasitenk., **99**, 25–46.

KORTENHAUS, F. 1929 Zonenzyklohämolyse auf Blutagar bei Staphylokokken sawie bei Viridans—Streptokokken und Pneumokokken. Zentr. Bakt. Parasitenk., **113**, 499–503.

DEKRUIF, P. H. 1922 Mutation of the bacillus of rabbit septicemia. J. Exptl. Med., **35**, 561–574.

KULP, W. L., LACKMAN, D., AND BORDEN, A. G. 1940 Non-specific immunization with members of the *Proteus* group. Unpublished data. Department of Bacteriology, University of Connecticut.

LEHMAN, K. B., AND NEUMANN, R. G. 1931 Determinative Bacteriology. Edited by R. S. Breed. G. E. Stechert and Company (Alfred Hafner) New York, 474.

LEIFSON, E. 1930 A method of staining bacterial flagella and capsules together with a study of the origin of flagella. J. Bact., **20**, 203–211.

Medical Research Council. 1929 A system of bacteriology in relation to medicine. His Majesty's Stationery Office, London, **4**, 308.

MEYER, K. F., NEILSON, N. M., AND FUSIER, M. L. 1921 Experimental typhoid-paratyphoid carriers. IV. A comparative study of the infections produced by intravenous injections of typhoid, paratyphoid A and B bacilli in normal and immunized rabbits. J. Infectious Diseases, **28**, 408–455.

MINNING, W., AND RITTER, G. 1937 Zur Kenntnis der Biologie und Systematik von Proteus und Kloakenbakterien (Kloacinae Haupt). Zentr. Bakt. Parasitenk., **140**, 27–35.

MOLTKE, O. 1927 Contributions to the characterization and systematic classification of *Bacillus proteus vulgaris* (Hauser). Monograph. Levin and Munksgaard, Copenhagen.

MOLTKE, O. 1929 Untersuchungen über das Phänomen des Schwarmens der Proteus-Bazillen. Zentr. Bakt. Parasitenk., **111**, 399–403.

MURRAY, I. J. 1916 The oxygen requirements of biological soil processes. J. Bact., **1**, 597–614.

RHODES, G. B. 1938 Satellite hemolytic zones in blood agar staphylococcus cultures. J. Infectious Diseases, **62**, 124–128.

ROGER, M. 1893 Une epizootic observée ches des grenouilles. Compt. rend. soc. biol., **5**, 709–711.

RUSSELL, F. H. 1898 An epidemic septicemic disease among frogs due to the *Bacillus hydrophilus fuscus*. J. Am. Med. Assoc., **30**, 1442–1449.

SANARELLI, G. 1891 Ueber einen neuen Mikro-organismus des Wassers, welcher für Thiere mit veränderlicher und constanter Temperatur pathogen ist. Zentr. Bakt. Parasitenk., **9**, 193–199.

Taylor, J. F. 1928 *B. proteus* infections. J. Path. Bact., **31**, 897–915.
Topley, W. W. C. 1926 The third Milroy lecture on experimental epidemiology. Lancet **1**, 645–651.
Topley, W. W. C., and Wilson, G. S. 1936 The Principles of Bacteriology and Immunity. William Wood & Company, Baltimore, Md. 2d ed.
Trambusti, A. 1893 Ueber die physiologische Wirkung der Stoffwechselprodukt des Hydrophilus Fuscus. Beitr. path. Anat., **14**, 317–336.
Webster, L. T. 1924 The epidemiology of a rabbit respiratory infection. V. Experimental snuffles. J. Exptl. Med., **40**, 117–127.
Weldin, J. C. 1926–27 The colon-typhoid group of bacteria and related forms. Relationships and classification. Iowa State Coll. J. Sci., **1**, 151.
Weldin, J. C., and Levine, M. 1923 An artificial key to the species and varieties of the colon-typhoid or intestinal group of bacilli. Abstracts Bact., **7**, 13.
Wolf, C. G. L. 1918–19 Contributions to the biochemistry of pathogenic anaerobes. VII. The biochemistry of *Bacillus proteus*. J. Path. Bact., **22**, 289–307.
Zinsser, H., Enders, J. F., and Fothergill, L. D. 1939 Immunity Principles and Applications in Medicine and Public Health. The Macmillan Company, New York, 164.

THE GAS RATIO AND SOME CORRELATED DISTINGUISHING PROPERTIES OF BACTERIA OF THE GENUS PROTEUS

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Hauser (1885) first used the name *Proteus* to describe three organisms which he termed *Proteus vulgaris*, *Proteus mirabilis*, and *Proteus zenkeri*. He described these organisms mainly on their morphological characteristics and gelatin liquefaction. In reporting a study of 84 *Proteus* cultures, Wenner and Rettger (1919) suggested that a division of the genus *Proteus* into species other than *Proteus vulgaris* and *Proteus mirabilis* was unsound, owing to the inconsistent reactions of these bacteria on media other than maltose. Bengtson (1919) studied a number of *Proteus* cultures biochemically and serologically, and concluded that the group had one well defined species, *P. vulgaris* and a number of other related species. She emphasized that laboratory stock cultures of *Proteus* often lost their power to ferment certain carbohydrates. As all of her cultures had been isolated for a considerable period before the study was conducted, Bengtson stated that a study of freshly isolated *Proteus* organisms should prove valuable. Studies by Weldin (1927), Yacob (1932) and Mello (1938) have also added to our present definition of the genus *Proteus*.

The significance of differences in gas production by bacteria for establishing natural species was recognized by Theobald Smith (1893). As a means of detecting gas production by bacteria he introduced the bent tube. Smith recognized that CO_2 was much more soluble in his medium than was H_2, and therefore concluded that his analyses for CO_2 must have been low. He was convinced, however, that gas production by bacteria would reveal phylogenetic relationships often not detectable by other tests, and he showed that gas production was one of the more permanent characteristics of bacteria. One of Smith's cultures of *P. vulgaris* which had almost completely lost its peptonizing power continued to give the same gas ratio value in the fermentation of glucose.

Keyes (1909) recognized the inaccuracies inherent in the analyses of gases collected in the Smith fermentation tube, and introduced a more accurate method for the collection and analysis of gases evolved during bacterial growth. He showed that differences existed in the production of gases by members of the coli-aerogenes group, but made no use of his results in the identification and classification of bacteria.

Rogers, Clark and Davis (1914) made further refinements on Keyes' method and demonstrated that by the gas ratio (CO_2/H_2), the colon bacteria could be separated into a well defined "low ratio" group (CO_2/H_2 = 1/1 approximately) and a distinct "high ratio" group (CO_2/H_2 = 1.5–2.8/1 approximately), and that the "high ratio" group was not as constant in the ratio value as the "low ratio" group. One culture was found which produced only CO_2. In a later

paper Rogers, Clark and Evans (1914) showed that the colon organisms isolated from bovine feces had a ratio of 0.98 to 1.20. They concluded that the "low ratio" group included those members of the colon group usually designated as *Escherichia coli* (*coli communis* of Sternberg) and *Escherichia coli*, var. *communior*, while the "high ratio" group included *Aerobacter aerogenes, Escherichia coli* var. *acidilacti* and possibly *Aerobacter cloacae.*

Rogers, Clark and Evans (1915) isolated seven colon-like bacteria which produced only CO_2; these were found among their 166 cultures isolated from grains. The seven organisms liquefied gelatin, failed to produce indole, and formed a yellow pigment on agar. They usually fermented sucrose, mannitol, glycerol, and sometimes adonitol, but usually failed to ferment lactose, raffinose, inulin, starch, and dulcitol.

Rogers (1921), developing a phylogenetic relationship of the colon-aerogenes bacteria, considered *Aerobacter cloacae* to be the organism from which other members of the group had developed. *Aerobacter aerogenes* had lost proteolytic activity. *E. coli* was still further removed by the loss of the acetylmethylcarbinol production and the development of indole formation. Removed from *A. cloacae* in the opposite direction was *Proteus vulgaris* which had lost in fermentation of carbohydrates—both in number fermented and the extent of fermentation—and had developed proteolytic activity. A small group of organisms, which liquefied gelatin, fermented glucose with the formation of CO_2 only, and fermented sucrose, but usually failed to ferment lactose, Rogers considered to be identical with *P. vulgaris.* It was found that the failure to ferment lactose, usually considered a distinguishing characteristic of *P. vulgaris,* did not coincide perfectly with the more fundamental character indicated by the production of only CO_2 in glucose fermentation. Furthermore, the unusual colony formation commonly looked upon as peculiar to *P. vulgaris,* was found to be shared with some *A. cloacae* cultures. Rogers postulated that a more logical separation between *A. cloacae* and *P. vulgaris* would be on the end-products of fermentation rather than on the nature of the material fermented. The amount of gas formed from glucose by the *Proteus* group was usually much less than that obtained even from the "low ratio" cultures.

Stable and well defined characteristics of bacteria have been the constant need of bacteriologists. Many groups of bacteria often exhibit considerable variation and at times vary in what may have appeared to be stable properties. The relationship of the genus *Proteus* to other apparently closely related groups of bacteria has not been satisfactorily explained. Many years ago L. A. Rogers and his associates clearly defined *Escherichia* and *Aerobacter* organisms by using the relative amounts of different gases produced from glucose. This important and fundamental work has been universally accepted without further confirmation, but in so far as is known by us, gas ratio studies have not been employed by more recent workers in their investigations of the gram-negative non-spore-producing rods. In the report here presented the results obtained from gas ratio determinations on a limited number of *Escherichia* and *Aerobacter* cultures, and on a larger number of *Proteus* cultures are reported.

METHODS

Cultures studied

The organisms studied were isolated from soil, street washings, creek water, raw sewage, sewage digest, and putrefying meat (table 8). From samples which contained sufficiently large numbers of *Proteus* organisms direct isolations were made. Samples containing small numbers of *Proteus* organisms were enriched by incubation for 24 hours at 30°C. in formate-ricinoleate broth (Stark and England, 1935). The plating medium was tryptone glucose skim-milk agar. Representative colonies were picked from the plates into nutrient broth, and after abundant growth was obtained, the culture was replated. One isolated representative colony was inoculated into nutrient broth and this purified culture was used for further study. Cultures were tentatively considered to be members of the genus *Proteus* when they produced acid and gas from glucose and usually from sucrose, failed to ferment lactose, and were gram-negative, non-spore-producing rods. Action on protein was not used as a primary differential characteristic but all cultures isolated by us, having the characteristics mentioned above, liquefied gelatin and rapidly peptonized litmus milk. Some of the organisms included in the study had been received from other laboratories. Each of these was plated and a representative colony picked into nutrient broth to insure the purity of the culture. This procedure may possibly have involved the selection of a variant of the original culture. The biochemical reactions of the purified and the original culture were, however, usually the same. A total of 108 cultures were included in this study, 66 of which were isolated by us and 42 of which were obtained from workers in other laboratories.

Collection and analysis of gases

The gases evolved by the bacteria were collected by an automatic Sprengel mercury pump and subsequently analyzed by methods very similar to those used by Rogers, Clark, and Davis (1914). Carbon dioxide was absorbed by KOH in a bubbling absorption pipette, and the hydrogen measured by slow combustion in pure oxygen. All measurements were made above mercury. As the gases were pumped from the culture cell they were forced through a chamber containing "dehydrite" to render them moisture-free. All gas volumes were reduced to 0°C. and 760 mm. of mercury.

In studies by previous workers no measurements were made of the carbon dioxide which had been produced and reacted with constituents of the medium. In a number of samples, during the present study, when the pH was found to be above 5.5, one ml. of concentrated lactic acid was added to the duplicate culture cell through the side arm of a three-way stopcock placed between the culture cell and the dehydrating chamber. In this way the acid could be added to the medium during the evacuation of the gases without allowing any air to enter. The CO_2 liberated by so lowering the pH (to about 2.5) was measurable, but had no significant effect on the ratio of CO_2/H_2. The amount of CO_2 liberated by this procedure was practically negligible when the pH was below 6.0.

The medium, unless otherwise stated, contained "Baker's Analyzed" or

Pfanstiehl's glucose, Witte peptone, and "Baker's Analyzed" K_2HPO_4 (dried powder). The per cent of the different ingredients was varied in some of the experiments and the formulae of the media are indicated in the respective tables. The formula used by Rogers and his co-workers was Witte peptone, 1.0 per cent; glucose, 1.0 per cent; K_2HPO_4, 0.5 per cent. This medium, when used in the studies reported here, was made by dissolving 10 gm. of Witte peptone and 5 gm. of K_2HPO_4 in 800 ml. of distilled water and the solution was then steamed for 20 minutes. After filtering off the precipitate, the solution was made up to volume, measured into the culture cells in 20 ml. amounts, and sterilized at 120°C. for 20 minutes. The glucose was sterilized by filtering a 5 per cent solution through a Seitz filter. Five ml. were added aseptically to the sterile medium which then had 1.0 per cent glucose. The pH of this medium was 7.5.

When the methyl-red medium was used, the ingredients were added in the following concentrations: Witte peptone, 0.5 per cent; glucose, 0.5 per cent; K_2HPO_4, 0.5 per cent. This medium was prepared essentially as Rogers' medium, except that the peptone and buffer were made to 900 ml., measured into the cells in 22.5 ml. amounts, and 2.5 ml. of sterile 5 per cent glucose added. The pH of the sterile medium was 7.6.

Twenty-five ml. amounts of medium, rather than 10 ml. as used by Rogers and co-workers, were used for a two-fold purpose. First, the larger volume of medium would give a larger sample of gas and make possible more accurate gas analyses. Second, *Proteus* organisms often do not produce as much gas as *Escherichia* and *Aerobacter*, and 25 ml. were needed in many instances in order to obtain an appreciable sample of gas.

The culture cells, for growing the organisms under vacuum, were made from 1¾ inch pyrex glass tubing to which was sealed a ⅜ inch outlet tube. Through this tube the medium was inoculated and then evacuated. Prior to evacuation the stem was constricted near the top, and after evacuation the cell was hermetically sealed at the constriction. The volume of the cells averaged approximately 200 ml.

The organisms to be tested were grown for at least two consecutive 24-hour periods before being inoculated into the culture cells, the first transfer being in nutrient broth and the last transfer to a nutrient agar slant. The growth on the slant was suspended in approximately 3 ml. of sterile distilled water, and 0.1 ml. of the suspension was inoculated into the culture cell. The inoculations were made soon after the medium was sterilized and cooled, so as to permit only a minimum amount of oxygen to dissolve in the medium. The cultures were incubated at 30°C. for 7 days and with a few exceptions were tested in duplicate.

Biochemical reactions

The sugars tested were sterilized by a Seitz filter and added to sterile nutrient broth to give approximately a 1 per cent solution of the sugar. The ability to use citrate as the only source of carbon was tested using the citrate broth developed by Koser (1923), and incubating for 2 days at 30°C. Acetylmethylcarbinol production was determined using the modified procedure of O'Meara

(1931) and by using a warm solution of 30 per cent KOH rather than the 40 per cent solution used by O'Meara. Four per cent nutrient gelatin was used for the gelatin liquefaction studies. Other tests were performed as recommended by the "Manual of Methods for Pure Culture Study of Bacteria" (1937). The media were incubated at 30°C. for 12–14 days (except the citrate broth, which was held only 2 days).

RESULTS

Effect of kind of peptone

One of the primary interests in the beginning of this study was to verify by our methods the well established ratios obtained by Rogers and his associates for the *Escherichia* and *Aerobacter* organisms. It was significant that, in order to duplicate their results for *Escherichia* bacteria, it was essential to use Witte

TABLE 1

The effect of the kind of peptone on the gas ratio of Escherichia, Aerobacter, and Proteus cultures (1.0 per cent peptone, 1.0 per cent glucose, 0.5 per cent K_2HPO_4)

CULTURE	PEPTONE	TOTAL GAS	CO_2	H_2	CO_2/H_2	FINAL pH
		ml.	*ml.*	*ml.*		
G71. *E. coli*	Bacto	41.95	23.69	16.47	1.44	4.60
G71. *E. coli*	Witte	29.65	15.86	12.59	1.26	4.36
11a10. *E. coli*	Bacto	36.16	19.89	14.03	1.42	4.63
11a10. *E. coli*	Witte	32.78	16.89	15.27	1.11	4.47
A67. *A. aerogenes*	Bacto	85.97	61.89	22.29	2.77	6.59
A67. *A. aerogenes*	Witte	66.40	48.80	17.07	2.88	4.77
Cb1. *A. aerogenes*	Bacto	76.57	54.95	19.87	2.77	6.31
Cb1. *A aerogenes*	Witte	63.67	44.19	18.60	2.35	5.31
52. *Proteus*	Bacto	24.61	12.28	8.85	1.39	4.82
52. *Proteus*	Witte	18.05	9.09	8.03	1.13	4.95
86. *Proteus*	Bacto	28.61	14.96	12.80	1.17	4.87
86. *Proteus*	Witte	23.09	11.79	10.55	1.12	4.96

peptone in the medium as formulated by them. When Bacto-peptone was used the *Escherichia* cultures gave ratios approximating those normally given by *Aerobacter* (table 1). It was interesting to find that using Bacto-peptone an appreciably larger volume of gas was obtained than when Witte peptone was used. These results are comparable with those reported by Burton and Rettger (1917) who found that more sugar was utilized in a medium containing Bacto-peptone than in one containing Witte peptone. These workers found that the sugar utilization was directly related to the amino-acid content of the medium. The results of the present study confirm those of Burton and Rettger since with Bacto-peptone a larger volume of gas was liberated. Although the CO_2 was mostly responsible for the increase in the total volume of gas from the Bacto-peptone medium, the volume of H_2 was also usually greater. This increase in CO_2 was probably only partly accounted for by the increased utilization of sugar. Considering the higher amino acid content of the Bacto-peptone, it was probable

that much of the increased CO_2 was derived from decarboxylation of the amino acids present. This assumption is supported by the work of Treece (1928) who studied gas production from commercial peptones by *A. aerogenes* and *E. coli*. His results showed that these two organisms produced gas from Difco but not from Witte peptone, and that most of the gas was CO_2. In order to study the gases produced from glucose by the action of the bacteria, it was necessary to use a peptone from which no gases were produced. In subsequent work on gas ratios, therefore, Witte peptone was used.

The effect of sugar concentration

The methyl-red test proposed by Clark and Lubs (1915) for the differentiation of the colon-aerogenes organisms was based on the final pH attained by the different organisms grown aerobically in a special glucose medium. These workers found that in a medium containing 0.5 per cent Witte peptone, 0.5 per cent K_2HPO_4 (anhydrous), and 0.5 per cent glucose, *Escherichia* organisms reached a low final pH which remained constant. *Aerobacter* organisms, however, did not maintain a low final pH, but were able to ferment the organic acids with a reversion of the pH to 6.0 or above. Since the *Aerobacter* cultures used in this study sometimes did not revert the pH in Rogers' medium (1.0 per cent glucose, 1.0 per cent Witte peptone, 0.5 per cent K_2HPO_4) it seemed logical to compare the gases produced in the methyl-red medium (0.5 per cent glucose), which permitted the fermentation of the organic acids by *Aerobacter*, (Ayers and Rupp, 1918) with the gases produced in Rogers' medium. In addition, gas ratios using the methyl-red medium containing 0.2 per cent glucose instead of the usual 0.5 per cent glucose were studied. For this comparative study two cultures each of *Aerobacter* and *Escherichia* and *Proteus* were used.

Comparing the methyl-red (0.5 per cent glucose) medium with the Rogers' medium, it was observed that the *Aerobacter* cultures reverted the pH normally in the methyl-red medium, and the inconsistent volumes of gas produced in the Rogers' medium by A67 were not observed in the methyl-red medium (table 2). The *Proteus* cultures frequently produced more gas in the methyl-red medium, which indicated that in the Rogers' medium the higher amount of sugar inhibited in some way the more active metabolism of these cultures. When the sugar content was reduced to 0.2 per cent, the *Aerobacter*, *Escherichia*, and *Proteus*, cultures produced nearly equal amounts of gas, and the gas ratios for all cultures were approximately 1/1. With this medium the pH was always reverted by all of the cultures, although it was never lower than approximately 6.0. This was shown by determining pH-time curves for *Escherichia*, *Aerobacter*, and *Proteus* cultures grown aerobically in media containing 1.0 per cent, 0.5 per cent, and 0.2 per cent glucose. These results showed that 0.2 per cent glucose was insufficient for the *Escherichia* cultures to produce the necessary amount of acids to maintain a low final pH.

In line with the important findings of Ayers and Rupp (1918), these results indicated that a medium could be chosen which would allow two very distinct and characteristic types of glucose dissimilation to proceed, depending upon the

organisms used. *Escherichia* cultures in a medium containing sufficient glucose produced more acids than they could decompose, and the pH of the medium remained low. *Aerobacter* cultures required a concentration of glucose which would not provide a source of more organic acids than they could utilize, but which would be sufficient to allow the characteristic secondary "alkaline" fermentation, as explained by Ayers and Rupp (1918), to proceed. The methyl-

TABLE 2

The effect of sugar concentration on the gas ratio

CULTURE	PER CENT GLUCOSE	TOTAL GAS	CO_2	H_2	CO_2/H_2	FINAL pH
		ml.	*ml.*	*ml.*		
11a10. *E. coli*	1.0(R)†	32.78	16.84	15.27	1.10	4.47
	0.5(MR)‡	29.60	16.56	16.23	1.02	4.60
	0.2(MR)	18.31	7.86	9.58	.82	6.49
G71. *E. coli*	1.0(R)	29.65	15.86	12.59	1.26	4.41
	0.5(MR)	37.87	20.10	17.46	1.15	4.97
	0.2(MR)*	23.40	8.85	8.67	1.02	6.65
A67. *A. aerogenes*	1.0(R)	7.91	6.59	.70	9.40	4.72
	1.0(R)	18.91	11.13	3.14	3.55	4.82
	1.0(R)	66.40	48.80	17.07	2.89	4.71
	0.5(MR)	33.50	21.78	10.23	2.13	6.48
	0.2(MR)	18.05	7.35	7.44	.99	6.35
Cb1. *A. aerogenes*	1.0(R)	63.67	44.19	18.60	2.38	5.31
	0.5(MR)	38.30	24.24	13.11	1.85	6.41
	0.2(MR)*	20.99	10.01	9.66	1.04	6.60
42. *Proteus*	1.0(R)	16.88	8.46	7.62	1.11	5.03
	0.5(MR)	28.12	13.66	14.10	.97	5.99
	0.2(MR)*	18.21	8.83	8.99	.98	6.50
100. *Proteus*	1.0(MR)	25.14	13.84	10.51	1.32	4.84
	0.5(MR)	23.98	12.56	11.01	1.14	5.17
	0.2(MR)*	19.64	9.62	10.02	1.04	6.63

* Lactic acid added to duplicate sample to liberate combined CO_2 and the gases thus obtained are reported.

† Rogers' medium (1.0 per cent glucose, 1.0 per cent Witte peptone, 0.5 per cent K_2HPO_4).

‡ Methyl-red medium containing amount of glucose as shown.

red medium of Clark and Lubs (1915) was found to be best for these purposes and was employed in the greater part of this study.

Gas ratios produced by freshly isolated Proteus cultures

On the basis of the gas ratio, it was observed that *Proteus* cultures formed three distinct groups (table 3). Group I was characterized by: a high gas ratio, the average being CO_2/H_2 = 6.28/1; a small total amount of gas produced, the average being 12.26 ml.; and a low final pH, the average being 4.97. Group II

produced: an intermediate ratio, the average being $CO_2/H_2 = 1.60/1$; a large total amount of gas, the average being about 35.23 ml.; and a high final pH, the average being 6.01. Group III was characterized by: a low gas ratio, the average being $CO_2/H_2 = 1.05$; a moderate amount of gas, the average being 20.29 ml.; and a relatively low final pH, the average being 5.22.

Group I may be identical with the eight cultures that Rogers (1921) termed the infinity ratio group (producing only CO_2) and considered to be the typical *Proteus* organism. It should be noted, however, that the gas analyses by Rogers and his associates were made on the gases produced from 10 ml. of medium. In view of the very small amount of hydrogen usually found to be formed by this group, in the present investigation, it may be that the volume of hydrogen from

TABLE 3

The gases and final pH produced from glucose by freshly isolated Proteus cultures grown under anaerobic conditions

GROUP	TOTAL CULTURES	TOTAL TESTS	CO_2/H_2	CO_2/H_2 RANGE	TOTAL GAS	FINAL pH
					ml.	
I	26	62	6.28	(2.50–14.54)	12.26	4.97
II	23	46	1.60	(1.31– 1.84)	35.23	6.01
III	17	28	1.05	(.90– 1.18)	20.29	5.22

Note: The medium contained 0.5 per cent each of glucose, Witte peptone, and K_2HPO_4; the cultures were incubated 7 days at 30°C.

TABLE 4

The gases produced by A. cloacae from glucose (methyl-red medium)

CULTURE	TOTAL GAS	CO_2	H_2	CO_2/H_2	FINAL pH
	ml.	*ml.*	*ml.*		
Mj2	33.93	22.46	10.91	2.06	5.88
Mj5	31.85	20.73	10.44	1.99	6.00
Mk5	20.52	13.57	6.62	2.04	6.20
Mk8	27.59	19.59	7.40	2.65	6.39

10 ml. might be too small to be measured. The small amount of hydrogen produced by some cultures caused the ratio to vary considerably from the average value, although there was little doubt about their belonging to this group.

Group II showed less variability in the gas ratio than group I. The reversion of pH, considered along with the large volume of gas produced, indicated that an entirely different type of fermentation was operating. One might, from these data, assume that this group of bacteria belonged to the *Aerobacter* genus. A comparison of the ratios obtained from several freshly isolated *A. cloacae* cultures, which shows the closest relationship biochemically to this group of *Proteus*, showed that the *Aerobacter* cultures produced an appreciably higher ratio than the *Proteus* cultures (table 4).

The gas ratio of group III was found to be that which is characteristically given

by *Escherichia* organisms. The final pH, however, was not so low as that usually produced by *Escherichia* organisms. Other differences will be considered in the section on biochemical characteristics.

Gas ratios of Proteus stock cultures

The gases produced by 42 cultures, most of which had been grown on laboratory media for a number of years and which were received from various laboratories in this country, were also studied (table 5). The medium used in the greater part of this study was that used by Rogers and his associates, since at the time of these studies the effect of the sugar concentration had not been determined by us. The ratios obtained using this medium were, however, not significantly different from those obtained when the methyl-red medium was used, as was shown by comparing the ratios given by 16 cultures tested on both media.

Thirty-eight of the 42 cultures gave a ratio value which would assign them to the low ratio group (table 3) formed by the fresh cultures. Most of these cultures gave an average of 21.69 ml. of gas, which was comparable in volume to

TABLE 5

The gases and final pH produced from glucose by old stock cultures of Proteus under anaerobic conditions

GROUP	TOTAL CULTURES	TOTAL TESTS	CO_2/H_2	CO_2/H_2 RANGE	TOTAL GAS	FINAL pH
					ml.	
I	1	4	4.71		22.04	5.85
II	3	6	1.69	(1.58–1.78)	33.04	5.31
III	38	119	1.07	(.97–1.30)	19.57	5.18

that given by the low ratio group of freshly isolated cultures. These results were interpreted as an indication that the organisms' mechanism for the dissimilation of glucose had not been impaired during laboratory cultivation. The final pH values compared favorably with those given by the fresh cultures. Three of the cultures belonged to group II. The total volume of gas produced averaged 33.04 ml. and the average ratio $CO_2/H_2 = 1.69$. That the average final pH was as low as 5.31 was probably accounted for by the fact that two of the cultures were tested on Rogers' medium and were about pH 5.0; the one culture tested on the methyl-red medium gave a final pH of 5.90, which agrees with the average value given by the fresh cultures. One culture was found to give typical values for organisms belonging to group I, although the final pH value was higher than normally would be expected.

The results obtained from these 42 cultures were considered as verifying evidence for the existence of the three groups found among the fresh cultures. It may also be assumed that laboratory cultivation does not affect the gas ratio characteristic of the organism, although definite knowledge of this must await future study.

Biochemical properties of freshly isolated Proteus cultures

It was noted in the foregoing sections that freshly isolated cultures of *Proteus* formed three distinct groups on the basis of the gas ratio (CO_2/H_2). In these groups the methyl-red test, the production of acetylmethylcarbinol, indole, and citrate utilization correlated with the gas ratio and final pH in the glucose medium used for the gas analyses (table 6). All of the cultures liquefied gelatin and peptonized litmus milk. The sugars which are used for classifying species of the genus *Proteus* (Bergey's Manual, 1939) were fermented by nearly all of the cultures and were found in this study to be of no real value for classification purposes.

All of the cultures in the high ratio group (CO_2/H_2 = 6.28) were methyl-red negative and all but two failed to produce indole. The infinity ratio group (produced CO_2 only) of Rogers, Clark and Evans (1915) also usually failed to produce indole, which is a further indication that this group of organisms may be that described by Rogers and his associates as the "infinity" group. Other

TABLE 6

Correlating properties of freshly isolated Proteus cultures

GROUP	SUGGESTED NAME	TOTAL CUL-TURES	AVERAGE RESULTS			M.R.	V.P.	INDOLE	CITRATE
			CO_2/H_2	Total gas	Final pH				
				ml.					
I	*P. hydrophilus*	26	6.28	12.26	4.97	−	+	−(24) +(2)	+ (24) − (2)
II	*P. mirabilis*	23	1.60	35.23	6.01	−	+	+	−
III	*P. vulgaris*	17	1.05	20.29	5.22	−(13) +(4)	−(14) +(3)	+	−

correlating properties were the production of large amounts of acetylmethylcarbinol and rapid growth in citrate broth.

The cultures belonging to the intermediate ratio group (CO_2/H_2 = 1.60) were distinctly different biochemically by their production of indole and by failing to grow readily in citrate broth. There are similarities in the gas ratio and final pH of this group to those given by *Aerobacter* organisms, but *A. cloacae* to which this group shows the most similarity, ferments lactose, grows readily in citrate broth, fails to peptonize litmus milk, and fails to produce indole (Bergey's manual, 1939). The gas ratio of this group of *Proteus* is also lower than that of the typical *A. cloacae* cultures (table 4). It would seem, therefore, that a second group of *Proteus* organisms exists which gives an average gas ratio of 1.60, produces a final pH of about 6.01 anaerobically in 0.5 per cent glucose broth, produces indole, and fails to utilize citrate readily as a sole carbon source.

Cultures belonging to the low ratio (CO_2/H_2 = 1.05) group produced indole and failed to grow readily in citrate broth, a few were methyl-red positive and a few others were V.P. positive. Although the reactions in the methyl-red and Voges-Proskauer tests were not as constant as in the other two groups, both tests

usually were negative. One culture produced acid and a small amount of gas in lactose broth. These are some of the usual characteristics of *Escherichia* cultures. However, the *Proteus* cultures were proteolytic in litmus milk and gelatin, and failed, with one exception, to ferment lactose. Since the possession of these properties is considered foreign to *Escherichia* cultures, and considered as identifying the genus *Proteus*, this third group of *Proteus* organisms is suggested on the basis of the gas ratio and other correlating characteristics.

An interesting observation on some of these newly isolated cultures was an apparent feeble fermentation of lactose. This was observed mainly among newly isolated cultures and became apparent after about two weeks' incubation. These observations confirm those reported by other investigators (Rogers, Clark, and Evans, 1915; Rogers, 1921; Mello, 1938).

OLD LABORATORY STOCK CULTURES

In the three groups formed, from these cultures, on the basis of the gas ratio (table 7), there were also correlations with indole formation, citrate utilization

TABLE 7

Correlating properties of old stock cultures of Proteus

GROUP	SUGGESTED NAME	TOTAL CULTURES	AVERAGE RESULTS			M.R.	V.P.	INDOL	CITRATE
			CO_2/H_2	Total gas	Final pH				
				ml.					
I	*P. hydrophilus*	1	4.71	22.04	5.85	−	+	−	+
II	*P. mirabilis*	3	1.69	33.04	5.31	−	2+ 1−	2− 1+	−
III	*P. vulgaris*	38	1.07	19.57	5.18	−	30− 8+	26+ 12−	−

and acetylmethylcarbinol production, although these correlations were not so good as those existing in the group of newly isolated cultures. All of these cultures showed some activity in milk, either by peptonization or by producing a basic reaction as shown by litmus. Five of the cultures failed to liquefy gelatin, although two of them were definitely able to cause peptonization in litmus milk. Whereas nearly all of the newly isolated cultures fermented glucose, galactose, maltose, mannitol, and sucrose, among these 42 old cultures 10 failed to ferment maltose and only two fermented mannitol. Only one old culture gave the typical reactions of group I. Three cultures were in group II. There was a tendency for the characteristics to be the same as those given by the fresh cultures of group II, although the cultures lacked satisfactory uniformity of biochemical properties. The majority of the old cultures belonged to group III. Like the newly isolated cultures of group III, most of the cultures produced indole, failed to grow readily in citrate broth, and did not produce acetylmethylcarbinol.

Although the old and fresh cultures agreed in the gas ratio values and to a less degree in the production of acetylmethylcarbinol, indole and in utilization of

citrate, there were few relationships shown in the sugars fermented by the old and fresh strains. These facts suggest that the more stable characteristics of *Proteus* organisms would be the gas ratio, final pH in 0.5 per cent glucose medium, production of acetylmethylcarbinol and indole, and the utilization of citrate, rather than the fermentation of various sugars. Further work, however, on the relative stabilities of the gas ratio and other biochemical properties would be desirable.

SUGGESTED NOMENCLATURE FOR PROTEUS SPECIES

With the data of this investigation indicating that three species exist in the genus *Proteus*, the names appearing in table 6 are suggested. These familiar names are now used for species in this genus. They are chosen for the particular groups since the cultures of each group represent most closely what we recognize at present as the respective species suggested.

TABLE 8

Sources of fresh cultures and their distribution in the three ratio groups

SOURCES	TOTAL CULTURES	DISTRIBUTION OF THE 3 GROUPS:		
		Group I $CO_2/H_2 = 6.28$	Group II $CO_2/H_2 = 1.60$	Group III $CO_2/H_2 = 1.05$
Soil	1	1	0	0
Street-washing	8	8	0	0
Creek water	13	2	7	4
Raw sewage	3	0	3	0
Sewage digest	22	0	13	9
Putrifying meat	19	15	0	4
Total	66	26	23	17

DISCUSSION

The present investigation has confirmed the results of Rogers and his associates on the CO_2/H_2 ratio of *Aerobacter* and *Escherichia* organisms. The *Escherichia* organisms produced a ratio of approximately 1/1 and the *Aerobacter* organisms a ratio of approximately 2/1. It should be emphasized, however, that in order to exhibit these fundamental characteristics the composition of the medium used is of major importance.

The close relationship between the proteolytic, lactose-non-fermenting, gram-negative rods classified as the genus *Proteus* and the *Escherichia-Aerobacter* organisms has been assumed for a considerable time. It is not surprising, therefore, that the gas ratio which has been so important in establishing the *Aerobacter* and *Escherichia* genera has revealed interesting relationships within the *Proteus* group. The validity of the three groups existing on the basis of the gas ratio is increased by the correlations with the properties of indole formation, citrate utilization, and acetylmethylcarbinol production.

The old stock cultures produced volumes of gas and gas ratios comparable

with fresh cultures of the same group, which indicated that prolonged laboratory cultivation had not impaired the organism's mechanism for dissimilating glucose. Although it was impossible to ascertain the gas ratio for these old cultures when they were first isolated, Rogers and his associates found that the ratio for colon organisms remained relatively constant during laboratory cultivation. Similar results were observed in this laboratory for *Aerobacter* organisms.

The marked differences in fermentative powers of the fresh and old cultures were very interesting, especially in view of the work of others who have shown that *Proteus* organisms lose the power to attack certain substrates. Bengtson (1919) reported that *Proteus* cultures often lost their power to ferment sucrose, maltose, and mannitol. The main differences between the fresh and old cultures in this study were that only two of the old cultures fermented mannitol and 10 failed to ferment maltose; yet all of the fresh cultures fermented maltose and all but three fermented mannitol. Although it will require time to determine which of the properties the present fresh group might lose by prolonged laboratory cultivation, it would appear that the old cultures used in this study had lost in their ability to attack maltose and mannitol. It seems illogical, in view of such observations, that these same properties which have been found to be lost during laboratory cultivation should be desirable characteristics for distinguishing between species of the *Proteus* genus (Bergey's manual, 1939). Since most classification studies of the *Proteus* genus have been based on the properties of collections of cultures which were entirely or in part old cultures, it would seem that a revision of the genus based on the apparently stable properties of the organisms, such as the gas ratio, should be made. Since it has been shown that these same characteristics, which are the most useful in identifying coliform organisms, are equally important in describing valid species of *Proteus*, it would seem advisable to adopt these descriptive properties for *Proteus*. It is not improbable that future work may show some equally good characteristics which can be used for identifying *Proteus* organisms; nor may the collection of cultures studied in this work have included all species possibly existing in the genus *Proteus*. But it is our belief that the stable characteristics mentioned will meanwhile assist in taxonomic studies of the genus.

The ability of *Proteus* to ferment lactose has been discussed by various workers and no general agreement has been attained, although it is generally accepted that *Proteus* organisms are essentially lactose-negative. In the present investigation certain of the fresh cultures suggested a feeble fermentation of lactose by the presence of a bubble of gas and a slight lowering of the pH at the end of about 14 days. Granting that this time is longer than the usual length of incubation, slow lactose fermenters also are found among the *Escherichia* and *Aerobacter* groups. How one can justify an arbitrary selection of the length of incubation to determine whether or not a sugar is fermented, is at least subject to question. The results of this investigation lead the authors to the same conclusion which Rogers (1921) stated concerning *Proteus*: ". . . traces of gas and a slight change in the hydrogen ion concentration sometimes observed in lactose broth indicate that there may be a feeble fermentation of this sugar by those cultures classed

as lactose negative." In the present investigation the fresh cultures showed a much higher frequency of a feeble lactose fermentation than was observed among the old cultures. In view of these observations, and those of others, it should not be surprising when occasionally a freshly isolated culture of *Proteus* shows some indication of lactose fermentation after an extended incubation period.

An interesting observation was that none of the cultures in this study failed to produce some hydrogen. Those belonging to group I of the fresh cultures, however, produced relatively very little hydrogen, with several giving particularly small amounts. Whether or not these cultures may be identical with those which Rogers and his associates found to produce no hydrogen is difficult to deduce. Other workers, however, recognized as *Proteus* organisms those which produced hydrogen, as is noted when Rogers, Clark, and Lubs (1918) stated: "We have designated as *B. proteus* those organisms which produce no hydrogen. This is not compatible with the characteristics of several cultures which others have furnished to us as *B. proteus*, but it is based on data obtained with seven cultures in our collection from grain." It is significant that these workers also encountered *Proteus* cultures which produced hydrogen, although the ratios of these cultures are not given. This is in agreement with findings in the present investigation. Of the 42 old cultures supplied to us from other laboratories, 38 produced equal amounts of hydrogen and CO_2, which would indicate that many bacteriologists are often familiar with only the low ratio group. The present investigation, based on cultures freshly isolated, suggests that the three groups are fairly equally distributed in nature (table 8).

SUMMARY AND CONCLUSIONS

The gas ratio (CO_2/H_2) and certain other biochemical properties of 108 strains of *Proteus* cultures were determined. Sixty-six of the cultures were recently isolated from natural sources and 42 had been grown on laboratory media for a number of years.

The recently isolated cultures of *Proteus* formed three distinct groups on the basis of the gas ratio. The average ratio for each group was 6.28 for group I, 1.60 for group II, and 1.05 for group III. Correlating with the gas ratio values for each group were the final pH of the 0.5 per cent glucose medium used for gas production, indole production, growth in Koser's citrate broth, and the production of acetylmethylcarbinol. Each of the old cultures of *Proteus* also gave a gas ratio which was characteristic of one of the three groups formed by the fresh cultures, although 38 of the 42 belonged to group III. The three groups of old cultures tended to show the same correlations in the biochemical properties which correlated with the gas ratio in the fresh cultures.

The old and fresh cultures showed considerable disagreement in their ability to ferment certain carbohydrates, but showed similarities in their gas ratio and the production of indole, acetylmethylcarbinol, and growth in citrate broth.

It is suggested that the genus *Proteus* be revised to contain three species, viz. *P. vulgaris*, *P. mirabilis*, and *P. hydrophilus*.

LITERATURE CITED

Ayers, S. H., and Rupp, P. 1918 Simultaneous acid and alkaline fermentations from dextrose and salts of organic acids respectively. J. Infectious Diseases, **23**, 188–216.

Bengtson, I. A. 1919 The *Proteus* group of organisms with special reference to agglutination and fermentation reactions and to classification. J. Infectious Diseases, **24**, 428–481.

Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchins, A. P. 1939 Bergey's Manual of Determinative Bacteriology. The Williams & Wilkins Co., Baltimore.

Burton, L. V., and Rettger, L. F. 1917 Correlations in the colon-aerogenes group. J. Infectious Diseases, **21**, 162–195.

Clark, W. M., and Lubs, H. A. 1915 The differentiation of bacteria of the colon-aerogenes family by the use of indicators. J. Infectious Diseases, **17**, 160–182.

Hauser, G. 1885 Über fäulnissbacterien. F. D. W. Vogel, Leipzig.

Keyes, F. G. 1909 The gas production of *Bacillus coli*. J. Med. Research, **21**, 69–82.

Koser, S. A. 1923 Utilization of the salts of organic acids by the colon-aerogenes gorup. J. Bact., **8**, 493–520.

Mello, J. de Toledo. 1938 Sobre o genero *Proteus*. Anais Fac. Med. Univ. Sao Paulo, **14**, 247–268.

O'Meara, R. A. Q. 1931 A simple delicate and rapid method of detecting the formation of acetylmethylcarbinol by bacteria fermenting carbohydrate. J. Path. Bact., **34**, 401–406.

Rogers, L. A. 1921 Characteristics and distribution of the colon-aerogenes group. Univ. Wisconsin Studies Sci., no. 2, 76–103.

Rogers, L. A., Clark, W. M., and Davis, B. J. 1914 The colon group of bacteria. J. Infectious Diseases, **14**, 411–475.

Rogers, L. A., Clark, W. M., and Evans, A. C. 1914 The characteristics of bacteria of the colon type found in bovine feces. J. Infectious Diseases, **15**, 99–123.

Rogers, L. A., Clark, W. M., and Evans, A. C. 1915 The characteristics of bacteria of the colon type occurring on grains. J. Infectious Diseases, **17**, 137–159.

Rogers, L. A., Clark, W. M., and Lubs, H. A. 1918 The characteristics of bacteria of the colon type occurring in human feces. J. Bact., **3**, 231–252.

Smith, T. 1893 The fermentation tube with special reference to anaerobiosis and gas production among bacteria. Wilder Quarter-Century Book, Ithaca, N. Y., p. 187.

Society of American Bacteriologists. 1937 Manual of Methods for Pure Culture Study of Bacteria. Sixth ed., Leaflet 5, p. 10. Commission on standardization of biological stains. Geneva, N. Y.

Stark, C. N., and England, C. W. 1935 Formate-ricinoleate broth—a new medium for the detection of colon organisms in water and milk. J. Bact., **29**, 26–27.

Treece, E. L. 1928 Gas production from commercial peptones by *B. aerogenes* and *B. coli*. J. Infectious Diseases, **42**, 495–500.

Weldin, J. C. 1927 The colon-typhoid group of bacteria and related forms. Relationships and classification. Iowa State Coll. J. Sci., **1**, 121–197.

Wenner, J. J., and Rettger, L. F. 1919 A systematic study of the *Proteus* group of bacteria. J. Bact., **4**, 331–353.

Yacob, M. 1932 A cultural and serological study of certain strains of the *Proteus* group. Indian J. Med. Research, **19**, 787–815.

LUMINOUS BACTERIAL AUXANOGRAMS IN RELATION TO HEAVY METALS AND NARCOTICS, SELF-PHOTOGRAPHED IN COLOR

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This study was undertaken with a three-fold objective: first, to compare the effects of some diverse bactericidal and bacteriostatic agents, including metal cations, sulfonamide compounds, and narcotics, on the growth and luminescence of luminous bacteria; second, to obtain self-photographs in color showing the chief results; and third, in connection with the photographs, to measure the brightness of the light and express it in absolute units.

Poisons of various kinds are known to reduce the intensity of luminescence in essentially non-proliferating suspensions or masses of the bacteria (Beijerinck, 1889, 1891; Harvey, 1915; Harvey and Taylor, 1934; Hill, 1929, 1932; Taylor, 1932, 1934, 1936; Shoup, 1933; Shoup and Kimler, 1934; Korr, 1935; van Schouwenburg, 1938; van Schouwenburg and van der Burg, 1940; Johnson and Chambers, 1939; Johnson and Moore, 1941) and in some cases a stimulation of luminescence by low concentrations of the inhibitors has been noted (Taylor, 1934, 1936; van Schouwenburg, 1938). A stimulation of growth and luminescence in broth cultures by low concentrations of urethane and of sulfanilamide (Johnson, 1942) as well as the stimulatory effects of bacteriostatic compounds, in low concentration, for various species on agar (Lamanna, 1942) lend a renewed interest to the familiar biological problem of apparent stimulations caused by low concentrations of toxic agents in general.

The self-photographs provide an opportunity of making essentially direct metabolic pictures of the cultures, showing the degree of inhibition or of stimulation apparent in the brightness of luminescence. In color, the pictures might hold a special interest for those whose conception of the real appearance of luminescent cultures is based solely on verbal descriptions, necessarily inadequate, or on the more precise but less vivid data of the spectrophotometer (Barnard, 1902; Molisch, 1912; Eymers and van Schouwenburg, 1936, 1937). The absolute intensity of the luminescence is of interest both as a matter of record and in connection with the problem of photography by such dim light. In studies of luminous micro-organisms it is ordinarily not essential to measure the brightness of their light in absolute units, and such data have only rarely been given (Lode, 1904, 1906; Friedberger and Doepner, 1907; Harvey, 1925; Eymer and van Schouwenburg, 1937; Johnson and Lynn, 1941). Data of this kind have apparently never been given in connection with photographs. Thus, although some interesting pictures taken by the light of luminous bacteria have been published (Dubois, 1901; Barnard, 1902; Molisch, 1903) the exposure times stated are of little use except under the identical conditions of the experiment, and they furnish only a very rough idea of the absolute intensities involved.

METHODS

The method most satisfactory from the point of view of this study appeared to be some modification of Beijerinck's "auxanogram" (Beijerinck, 1889, 1891) which is essentially the same as the agar plate technique for testing disinfectants (U. S. Department of Agriculture, 1931).

The surface of a solid medium, consisting of 3 per cent NaCl nutrient agar in Petri dishes with porcelain covers, was inoculated with a suspension of cells from a luminous slant, either with the aid of a sterilized camel's hair brush, or simply by flooding the agar surface, allowing the excess fluid to evaporate or be

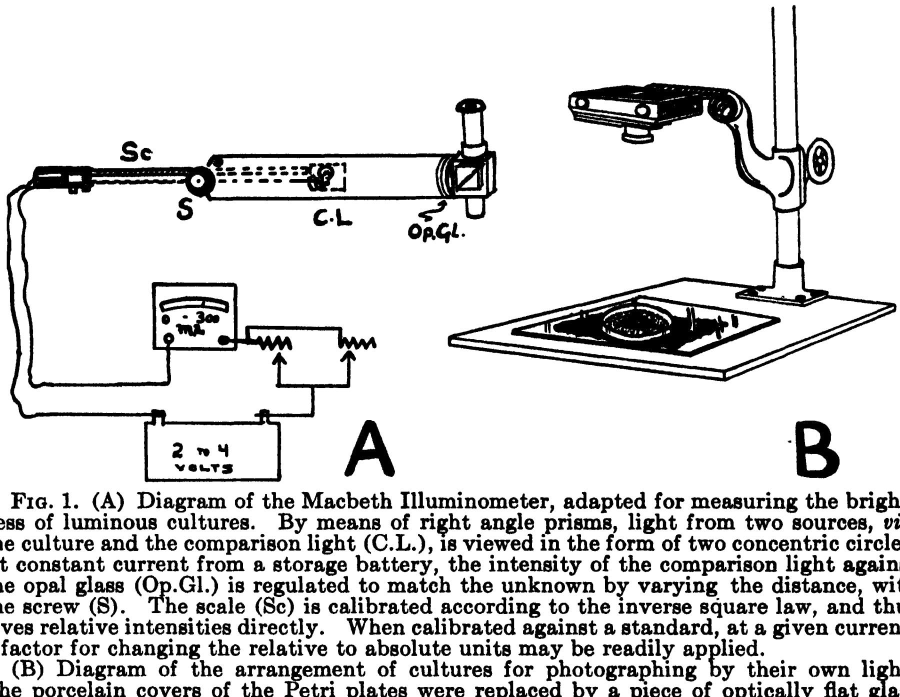

FIG. 1. (A) Diagram of the Macbeth Illuminometer, adapted for measuring the brightness of luminous cultures. By means of right angle prisms, light from two sources, *viz.* the culture and the comparison light (C.L.), is viewed in the form of two concentric circles. At constant current from a storage battery, the intensity of the comparison light against the opal glass (Op.Gl.) is regulated to match the unknown by varying the distance, with the screw (S). The scale (Sc) is calibrated according to the inverse square law, and thus gives relative intensities directly. When calibrated against a standard, at a given current, a factor for changing the relative to absolute units may be readily applied.

(B) Diagram of the arrangement of cultures for photographing by their own light. The porcelain covers of the Petri plates were replaced by a piece of optically flat glass during the long exposures necessitated by the dim light. A black cloth beneath the plate was necessary to prevent a double, or a blurred image.

absorbed into the medium and porous cover. A small amount of the test substance, usually only a few small crystals or particles, was then placed on the surface of the medium at the center, where it could diffuse peripherally, thus establishing a concentration gradient as the culture developed.

Preliminary experiments indicated that essentially the same results might be expected with any of several species, including both fresh water and marine types, cultivated under optimal conditions in each case. Consequently the organisms producing the brightest light with the least trouble were selected: *Photobacterium phosphoreum*,[1] a non-motile micrococcus growing best at 15°C.

[1] A culture of this species was obtained in 1939 from the Delft collection, through the kindness of Professor A. J. Kluyver.

and *Achromobacter fischeri* (Beijerinck) Bergey *et al.* (1939) a motile, mesophilic rod, with an optimum temperature of about 25°C.

The absolute light intensity given out by the cultures is at best far too weak to give a measurable voltage on a photronic cell of the Weston type. A photoelectric cell with vacuum tube amplifiers and sensitive galvanometer could be used, but visual photometry, with the aid of a modified Leeds and Northrup MacBeth Illuminometer, was just as sensitive and much more convenient in practice (fig. 1, A). At any constant current a range in intensities of 25 times could be matched and read directly from the scale. By calibrating the intensity of the illuminometer light at different amperages the range could be extended to cover from 1 to 25,000, and with the aid of neutral filters, intensities many times higher could be matched with the same instrument. The brightness of the cultures actually photographed, however, varied from approximately 0.002 to 0.04 millilamberts, corresponding to illuminations of 0.0018 to 0.036 foot-candles on a perfectly reflecting surface.

To match the quality of luminescence, two Wratten gelatin filters were placed in front of the opal glass in the illuminometer tube. The change in quality of the light emitted from the filament at different amperages, resulting from the change in spectral distribution of radiation at different temperatures, was made unnoticeable to the observer by these filters.

The photographs were made on Kodachrome "A" 35-mm. film in a Contax camera with f 2 lens (fig. 1, B).

RESULTS

The pictures in Plate I[2] show some typical results, and furnish a very good impression, by and large, of the true appearance of the luminescent cultures when seen by their own light.[3] Table 1 summarizes the data relating to these pictures. Certain points of interest, set forth below, call for special mention.

With substances which exert no effect, such as a small amount of NaCl, the luminescence is of practically uniform brightness over the surface of the medium (plate I, fig. 11). This result with NaCl is important in showing the absence of an effect of this anion. With active substances, the dark, circular area at the center of the culture represents the region in which growth was inhibited, and the brighter ring at its periphery represents a zone of stimulation. The interpretation of the latter will be considered later. With regard to the former, it is evident, from plate I and table 2, that many substances of diverse nature will prevent growth. The actual extent of the area of inhibition is obviously dependent upon a complex interaction of several variables, including the potency of the substance, its solubility, its rate of diffusion through the medium, and the rate of growth of the organisms. For example, the area of inhibition is greater when the same substance, e.g., copper, is added in the form of a soluble salt than when the metal itself is used (plate I, figs. 4 and 5). With the salt, it is greater when the inoculum

[2] The publication of this color plate was made possible through funds contributed for this purpose by an alumnus of Princeton University.

[3] The narrow, dull black circles immediately surrounding each figure are an artifact, introduced in the process of engraving.

is dilute, thus allowing inhibitory concentrations to diffuse further before the culture has developed (plate I, figs. 5, 6, and 7, of cultures inoculated with a suspension diluted in a ratio of 1, 1:3, and 1:5 respectively). Certain substances, with unusually great solubilities and relatively low potencies, or highly volatile substances such as ether, cannot be studied satisfactorily by this auxanographic method. The general results obtained with a variety of metals, metal chlorides, narcotics, *et cetera*, when added in the manner described at the time of inoculation

TABLE 1

Data concerning the photographs in plate I

Cultures of *A. fischeri* were incubated at 25°C., those of *P. phosphoreum* at 15°C. All pictures were taken at room temperature 23° to 25°C.

FIGURE	SUBSTANCE ADDED TO CENTER OF PLATE	SPECIES OF BACTERIA	AGE OF CULTURES	BRIGHTNESS	PHOTOGRAPHIC EXPOSURE-TIME
			hours	*micro-lamberts*	*minutes*
1	$CuCl_2$	P. phosphoreum	30	16.8	120
2	$CuCl_2$	P. phosphoreum	48	10.5	60
3	$CuCl_2$	P. phosphoreum (same plate as in fig. 2)	96	8.6	60
4	Cu	A. fischeri	20	4.3	105
5	$CuCl_2$	A. fischeri (Inoculum undiluted)	14	16.8	90
6	$CuCl_2$	A. fischeri (Inoculum diluted 1:3)	24	2.6	150
7	$CuCl_2$	A. fischeri (inoculum diluted 1:5)	24	1.7	150
8	$CuCl_2$	P. phosphoreum	48	14	60
9	p-Aminobenzoic acid	P. phosphoreum	24	16	120
10	$NiCl_2$	P. phosphoreum	48	13.4	60
11	NaCl	A. fischeri	24	34	25
12	Na-phenobarbital	P. phosphoreum	48	7.0	80
13	Glucose	P. phosphoreum	48	3.8	120
14	Glucose	A. fischeri	24	8.6	90
15	$NiCl_2$	P. phosphoreum	24	39.8	120
16	p-Aminobenzoic acid	P. phosphoreum	96	9.6	60
17	AgCl	P. phosphoreum	48	16.8	60
18	As	A. fischeri	24	5.8	100
19	$PbCl_2$	A. fischeri	24	6.5	100
20	$MnCl_2$	A. fischeri	14	7.6	90

or later, are summarized in tables 2 and 3. It should be noted that in most cases inhibitory effects in regions of high concentration are accompanied by some evidence of stimulation at low concentrations.

The zones of excess brightness might represent a stimulation of growth, or of luminescence, or of both. In some cases there was a visibly greater density of cells in such zones, but this difference was not always apparent. Oxidizable substrates are known to increase the intensity of luminescence much in the

manner that respiration may be increased without involving any considerable increase in numbers of cells (Johnson, 1936, 1938, 1939; van Schouwenburg, 1938; Johnson, van Schouwenburg and van der Burg, 1939). As mentioned above, certain narcotics in low concentration do likewise (Taylor, 1934, 1936; van Schouwenburg, 1938). Thus, while the intensity of luminescence under different

TABLE 2

Summary of luminous bacterial auxanograms of pure metals, metal chlorides, and narcotics

	SUBSTANCE	ZONE OF INHIBITION OR DECREASED BRIGHTNESS	ZONE OF INCREASED BRIGHTNESS
Metals	Aluminum	–	–
	Arsenic	++	+
	Bismuth	±	±
	Cadmium	++	±
	Cobalt	++	++
	Copper	++++	++++
	Gold	–	–
	Lead	+	+
	Manganese	±	+
	Magnesium	±	+
	Nickel	+	–
	Tin	±	–
	Zinc	+	–
Metal chlorides	$CoCl_2$	++++	++++
	$CuCl_2$	++++	++++
	$PbCl_2$	+	++
	$MnCl_2$	++++	++++
	$MgCl_2$	+	+
	NaCl	–	–
	$NiCl_2$	++++	+++
	$AgCl_2$	++	++
Narcotics et cetera	Benzamide	++++	++++
	Glucose	++++	++++
	p-Aminobenzoic acid	++++	++++
	Novocaine	+++	–
	Sulfapyridine	+++	++
	Sulfanilamide	++++	++++
	Sulfonmethane	+	+
	Sodium barbitol	+	+
	Sodium phenobarbital	++++	++++

– = none; + = slight, ++ = moderate, +++ = pronounced, ++++ = very pronounced.

conditions is not necessarily proportional to the number of cells, it does furnish a sensitive indicator for the net result—inhibition or stimulation—by the substance in question. Using the same procedure as with the inhibitors, the addition of glucose results in a zone of increased brightness that represents an additive effect on growth and on luminescent metabolism (plate I, figs. 13 and 14). The dark area in this case represents the area in which the glucose concentration was

high enough to give rise to sufficient acid production to retard growth and extinguish the light. Beijerinck (1891) was aware of this phenomenon. The addition of phenol red, or bromthymol blue showed that the region of increased brightness coincides with the zone of transition between the acid, non-luminous area and the approximately neutral, normal area.

The stimulatory effects of certain metallic cations, such as Cu, Ni, Co, and, Mn, are to some extent dependent on growth. The zone of stimulation of Cu for example, is much more pronounced when the inoculum is diluted, thus lengthening the normal time and number of "generations" preceeding maximum growth (plate I, figs. 5, 6 and 7; ratio of cells in inocula: 1, 1/3, 1/5, respectively). On the other hand, stimulation of luminescence by metallic ions in some cases

TABLE 3

Effects of metal chlorides, narcotics, etc., on luminescence of plate cultures of P. phosphoreum

Substances added at, and following, maximum growth

	CULTURES APPROXIMATELY AT MAXIMUM GROWTH AND LUMINESCENCE: 36 HRS. 15°C.						CULTURES WELL PAST MAXIMUM GROWTH AND LUMINESCENCE: 60 HOURS AT 15°C.					
	Time of observation after adding substance						Time of observation after adding substance					
	15–30 minutes		2½ hours		24 hours		15–30 minutes		1½ hours		22 hours	
	Inhibition	Stimulation	Inhibition	Stimulation	Inhibition	Stimulation	Inhibition	Stimulation	Inhibition	Stimulation	Inhibition	Stimulation
$CoCl_2$	+	−	++	−	+++	+	+	++	++	++	+++	++
$CuCl_2$	+	±	++	±	++	++	+	±	++	++	+++	++
$MnCl_2$	+	+	++	++	+++	++	+	+	++	++	+++	++
$NiCl_2$	+	±	++	++	+++	++	+	+	++	±	+++	++
Glucose	−	++	−	+++	+	+++	−	+++	+	+++	++	++
Benzamide	+	−	+	±	+	−	+	−	++	−	+++	−
p-Aminobenzoic acid	+	±	+	−	+	+	+	+	+	+	+	+
Sulfanilamide	+	±	+	−	+	−	+	−	+	−	+	−
Ethyl carbamate	+	−	++	−	++	−	+	−	++	±	++	−
Phenyl carbamate	+	±	++	+++	++	+						

− = none; + = slight, ++ = moderate, +++ = very pronounced.

appears to be a direct metabolic effect, since it appears in too short a time to be accounted for by increase in cell numbers, when the substance is added to either old or young cultures (table 3). It is noteworthy that this occurs with a non-motile species, thus excluding migration of the organisms as a possible explanation.

The narrow zones of excess brightness observed with metallic ions (plate I, figs. 1 to 8, 10, 15, 17 to 20) no doubt indicate a narrow range in the concentrations causing complete inhibition, a slight stimulation and no effect. With certain narcotics, e.g., phenobarbital (plate I, fig. 12) the zone of excess brightness is much wider, and the contrast between this and the normal area is less striking. Both the range of effective concentration and the rate of spread through

the agar probably are important factors. Temperature is an additional factor whose fundamental significance in inhibitions generally has recently been emphasized (Johnson, Brown and Marsland, 1942). In the present study cultivation and observations were carried out in so far as possible at the optimum temperatures of the particular organisms. With certain substances the relative difference in brightness between the normal and stimulated zones, however, could be reversibly altered in the same plate simply by equilibrating it at different temperatures. The influence of various factors on the temperature coefficient of luminescence intensity is a phenomenon of fundamental importance, and is under further investigation.

DISCUSSION

Although the results described above are purely qualitative they furnish an interesting survey of the bacteriostatic effects of a very diverse group of substances. Since growth is a complex process, the precise mode and site of action of the different types of inhibitors might well be different, and the evidence in this work does not justify more than general conclusions. However, inasmuch as practically every inhibition was accompanied by some evidence of increased luminescence in the region of low concentrations of the inhibitor, it is perhaps worth while to discuss these apparent stimulations somewhat further.

It might be argued that all cases of apparent stimulations of luminescence represent merely indirect effects resulting from differences in concentration of nutrients, or of waste products. At the edge of any zone of no-growth on agar there is always a concentration gradient of nutrient substrates and waste products respectively. In fact, a sterile cover-glass placed on the agar surface after inoculation was found to imitate very successfully the appearance of a zone of inhibition, surrounded by a narrow zone of excess luminescence. No growth occurred beneath the cover-glass for lack of sufficient oxygen. The excess brightness at the edges probably did not result from impurities in the glass, since a plastic cover-slip gave the same results. In this case, nutrients or metabolites might have been the chief factors in the stimulation, although others might also have been concerned, e.g., the gradient in oxygen tension, or the increased surface area at the edge of the glass.

Certain facts would indicate that the stimulations observed were real, i.e., not merely the indirect result of influencing the concentration of nutritive substrates in the medium itself. For example, many of the metallic cations increase the luminescence intensity in too short a time for the nutrient concentration in the adjacent region of the agar to assume any significance (table 3). Furthermore, as mentioned at the start, low concentrations of narcotics have been observed to stimulate bacterial luminescence under conditions not particularly favorable to growth (Taylor, 1934, 1936; van Schouwenburg, 1938). They may even increase the velocity of the luminescent oxidation completely apart from a living cell in crude extracts of the luminous Ostracod, *Cypridina* (Taylor, 1934). There is reason to believe, however, that in the crude extracts several simultaneous reactions may be concerned in the control of light emission (Anderson,

1936) and a stimulating effect by narcotics on the luminescent oxidation of highly purified luciferin and partially purified luciferase has not been observed (Johnson and Chase, 1942). These facts are all consistent with the usually accepted interpretation that stimulations of biological processes by low concentrations of poisons are due to inhibitions of some process or reaction limiting the velocity of the one stimulated.

An important experiment has been devised by van Schouwenburg (1938) indicating that bacterial luciferase is most probably an auto-oxidizable enzyme, practically insensitive to cyanide, and distinct from the cyanide-sensitive oxidase responsible for most of the aerobic respiration. This same experiment may be looked upon as a model for picturing stimulations that result indirectly from the inhibition of a competing reaction. The essential facts are as follows.

Under ordinary conditions of more or less complete aeration, the activity of neither the luciferase nor oxidase is limited by the amount of oxygen. At low oxygen tensions, however, the activity of both enzymes is reduced, and they are thrown into competition with each other for the amount of oxygen. Under these competitive conditions, any specific factor which would eliminate one enzyme should result in an increase in oxidizing activity of the other. Van Schouwenburg obtained striking evidence that this was actually the case by adding cyanide under conditions of low oxygen tension. The cyanide eliminated the oxidase from the competition for oxygen. There was an immediate, several-fold increase in luminescence when the CN was added. It would appear entirely reasonable to suppose that stimulatory effects resulting from low concentrations of diverse poisons might in general work in a similar manner, i.e., by inhibiting a competing system. If the competing system were concerned with growth, or pigment production, or any other integrative process, the results would be cumulative; i.e., a 10 per cent increase in rate of the process would soon result in a 100 per cent increase in total product. These considerations might be taken into account also in interpreting the bacteriostatic action of sulfonamide and other compounds, with particular reference to the antagonism of their action by diverse agents, such as p-aminobenzoic acid (Woods, 1940), methionine (Kohn and Harris, 1941) and urethane (Johnson, 1942; McIlwain, 1942). The weight of available evidence indicates that, in affecting the growth of bacteria, sulfanilamide and p-aminobenzoic acid act at the same site, in some specific, competitive manner. The latter compound, or a related one, may be normally involved in metabolic reactions essential to growth, or in any case, it allows certain reactions, susceptible to inhibition by sulfanilamide, to proceed in its presence. Substances other than p-aminobenzoic acid, however, e.g., urethane, which also antagonize the sulfanilamide inhibition, might exert their stimulatory effects by a different mechanism, perhaps analogous to that involved in the cyanide stimulation of luminescence at low oxygen tensions, as described above. The possibility remains, of course, that in some cases stimulation may occur through a direct effect on a specific enzyme system, as, for example, the well known influence of magnesium on phosphatase activity (Erdtman, 1927). In general, a more probable and understandable basis for stimulatory and antagonistic effects of

non-specific inhibitors, including metal cations, urethane, and in certain cases sulfanilamide and p-aminobenzoic acid also, would appear to consist in the slight inhibition of a reaction limiting the rate of growth or other process in question.

The authors wish to express their sincere appreciation for the contribution of funds by an alumnus to enable publication of the color plate. The authors are indebted to Professor Harvey for apparatus and suggestions. Mr. J. P. Warter Jr. also rendered valuable assistance through previous work in which he obtained probably the first successful color pictures taken solely by the light of luminescent cultures. Some technical information was kindly supplied by the Eastman Kodak Company.

SUMMARY

The effects of certain metals, metal chlorides, narcotics, and bacteriostatic compounds on the luminescence of plate cultures of *Achromobacter fischeri* and *Photobacterium phosphoreum* were studied by an auxanographic method.

Inhibitory effects on growth were caused by the addition of pure metallic copper, cobalt, cadmium and arsenic; to a slight degree by zinc, lead and nickel; to a doubtful extent by manganese, magnesium and bismuth; and not at all by aluminum or gold. Pronounced inhibitions were apparent with the chlorides of copper, cobalt, manganese, nickel and silver; and slight inhibitions by the chlorides of lead and magnesium.

Among organic compounds, pronounced inhibitions were found with benzamide, p-aminobenzoic acid, novocaine, sulfapyridine, sulfanilamide, and sodium phenobarbital. Sulfonmethane and sodium barbital were less effective. Glucose inhibited growth and luminescence because of the acid produced from it by the organisms.

In most cases, a zone of inhibition in the region of highest concentration was separated from the area of normal growth and luminescence by a zone of excess brightness. The interpretation of the two zones, especially the one of apparent stimulation, is discussed briefly in relation to certain factors and possible modes of action of the inhibitors. Certain substances, including both metal cations and narcotics, readily gave rise to zones of inhibition and stimulation, respectively, of luminescence when added to mature cultures.

The absolute brightness of luminescence was measured by a modified MacBeth Illuminometer, and found to range from 0.002 to 0.040 millilamberts at room temperature for cultures between one half and 4 days incubation at optimum temperature.

Color pictures of the plate cultures, taken by their own light, were obtained in order to show not only the qualitative characteristics of the luminescence, but its relative brightness in the different zones of the auxanogram.

REFERENCES

Anderson, R. S. 1936 Chemical studies on bioluminescence. III. The reversible reaction of Cypridina luciferin with oxidizing agents and its relation to the luminescent reactions. J. Cellular Comp. Physiol., 8, 261–276.

Barnard, J. E. 1902 Luminous bacteria. Nature, 65, 536–538.

Beijerinck, M. W. 1889 L'auxanographie, ou la methode de l'hydrodiffusian dans la gelatine appliquée aux recherches microbiologiques. Arch. neérland. sci., 23, 367–372.

Beijerinck, M. W. 1891 Sur l'aliment photogene et l'aliment plastique des bacteries lumineuses. Arch. neérland. sci., 24, 369–442.

Bergey et al. 1939 Manual of determinative bacteriology, 5th ed. The Williams & Wilkins Co.

Dubois, R. 1901 Das kalte Licht. Die Umschau, V. Jahrgang, No. 12, March 16, 221–224.

Erdtman, H. 1927 Glycerophosphatspaltung durch Nierenphosphatase und ihre Aktivierung. Z. physiol. Chem., 172, 182–198.

Eymers, J. G., and van Schouwenburg, K. L. 1936 On the luminescence of bacteria. I. A quantitative study of the spectrum of the light emitted by Photobacterium phosphoreum and by some chemiluminescent reactions. Enzymologia, 1, 107–119.

Eymers, J. G., and van Schouwenburg, K. L. 1937 On the luminescence of bacteria. II. Determination of the oxygen consumed in the light emitting process of Photobacterium phosphoreum. Enzymologia, 1, 328–340.

Friedberger, E., and Doepner, H. 1907 Ueber den einfluss von Schimmelpilzen auf die Lichtintensität in Leuchtbacterienkulturen nebst Mitteilung einer Methode zur vergleichenden photometrischen Messung der Lichtintensität von Leuchtbakterienkulturen. Zentr. Bakt. Parasitenk., I, 43, 1–7.

Harvey, E. N. 1915 The effect of certain organic and inorganic substances upon light production by luminous bacteria. Biol. Bull., 29, 309–311.

Harvey, E. N. 1925. The total luminous efficiency of luminous bacteria. J. Gen. Physiol., 8, 89–108.

Harvey, E. N., and Taylor, G. W. 1934 The oxygen consumption of luminous bacteria in water containing deuterium oxide. J. Cellular Comp. Physiol., 4, 357–362.

Hill, S. 1929 The penetration of luminous bacteria by the ammonium salts of the lower fatty acids. J. Gen. Physiol., 12, 863–872.

Hill, S. E. 1932 The effects of ammonia, of the fatty acids, and of their salts, on the luminescence of Bacillus Fischeri. J. Cellular Comp. Physiol., 1, 145–159.

Johnson, F. H. 1936 The aerobic oxidation of carbohydrates by luminous bacteria and the inhibition of oxidation by certain sugars. J. Cellular Comp. Physiol., 8, 439–463.

Johnson, F. H. 1938 Hexose oxidation by luminous bacteria. III. The escape of respiration and luminescence from inhibition by alpha methylglucoside. J. Cellular Comp. Physiol., 12, 281–294.

Johnson, F. H. 1939. Total luminescence of bacterial suspensions in relation to the reactions concerned in luminescence. Enzymologia, 7, 72–81.

Johnson, F. H. 1942 Mechanism of p-aminobenzoic acid action and the parallel effects of ethyl carbanate (urethane). Science, 95, 104–105.

Johnson, F. H., Brown, Dugald, and Marsland, Douglas 1942 A basic mechanism in the biological effects of temperature, pressure and narcotics. Science, 95, 200–203.

Johnson, F. H., and Chambers, E. L. 1939 Oxygen consumption and methylene blue reduction in relation to barbital inhibition of luminescence. J. Cellular Comp. Physiol., 13, 263–267.

Johnson, F. H., and Chase, A. M. 1942 The sulfonamide and urethane inhibition of Cypridina luminescence in vitro. J. Cellular Comp. Physiol., 19, 151–161.

Johnson, F. H., and Harvey, E. N. 1938 Bacterial luminescence, respiration and viability in relation to osmotic pressure and specific salts of sea water. J. Cellular Comp. Physiol., 11, 213–232.

Johnson, F. H., and Lynn, W. D. 1940–1941 The flash of luminescence following anaerobiosis of fungous mycelium. Anat. Record, 78, Supp. p. 65.

Johnson, F. H., and Moore, K. 1941 Sulfonamide inhibition of bacterial luminescence. Proc. Soc. Exptl. Biol. Med., 48, 323–325.

Johnson, F. H., van Schouwenburg, K. L., and van der Berg, A. 1939 The flash of luminescence following anaerobiosis of luminous bacteria. Enzymologia, 7, 195–224.

KOHN, HENRY I., AND HARRIS, J. S. 1941 Specific antagonism between methionine and sulfanilamide in *E. coli*. Am. J. Physiol. Proc., **133**, 354.

KORR, I. M. 1935 An electrometric study of the reducing intensity of luminous bacteria in the presence of agents affecting oxidations. J. Cellular Comp. Physiol., **6**, 181–216.

LAMANNA, C. 1942 Growth stimulation by sulfanilamide in low concentrations. Science, **95**, 304–305.

LODE, ALOIS 1904 Versuche, die optische Lichtintensitat bei Leuchtbakterien zu bestimmen. Zentr. Bakt. Parasitenk., I, Orig., **35**, 524–527.

LODE, ALOIS 1907 Experimente mit leuchtbakterien. Ber. deut. naturmedizinischen Vereins in Innsbruck, **21**, 23–24.

MCILWAIN, H. 1942 The biochemical specificity of sulfanilamide and of other antibacterial agents. Science, **95**, 509–511.

MOLISCH, H. 1903 Bakterienlicht und photographische Platte. Sitzber. Akad. Wiss. Wien, Math.-naturw. Klasse, **112**, 297–316.

MOLISCH, H. 1912 Leuchtende Pflanzen. Fischer, Jena.

VAN SCHOUWENBURG, K. L. 1938 On respiration and light emission in luminous bacteria. Thesis, Delft.

VAN SCHOUWENBURG, K. L., AND VAN DER BURG, A. 1940 On the influence of carbon monoxide on respiration and light emission of luminous bacteria. Enzymologia, **9**, 34–42.

SHOUP, C. A. 1933 Luminescence and respiration of bacteria in carbon monoxide. Biol. Bull., **65**, 370.

SHOUP, C. A., AND KIMLER, A. 1934 The sensitivity of the respiration of luminous bacteria for 2,4-dinitrophenol. J. Cellular Comp. Physiol., **5**, 269–276.

TAYLOR, G. W. 1932 The effects of hormones and certain other substances on cell (luminous bacteria) respiration. J. Cellular Comp. Physiol., **1**, 297–331.

TAYLOR, G. W. 1934 The effect of narcotics on respiration and luminescence in bacteria, with special reference to the relation between the two processes. J. Cellular Comp. Physiol., **4**, 329–355.

TAYLOR, G. W. 1936 The effect of ethyl urethane on bacterial respiration and luminescence. J. Cellular Comp. Physiol., **7**, 409–415.

U. S. Dept Agriculture 1931 U. S. Food and Drug Administration methods of testing antiseptics and disinfectants. Circ. 198.

WOODS, D. D. 1940 The relation of p-aminobenzoic acid to the mechanism of the action of sulfanilamide. Brit. J. Exptl. Path., **21**, 74–90.

PLATE I[2,3]

Plate Cultures of Luminous Bacteria Taken on Kodachrome Film by Their Own Light

The central dark areas are regions in which growth of the culture was inhibited by metallic ions, sulfanilamide, narcotics, or other substances, added to the agar surface at the time of inoculation, and allowed to diffuse peripherally as the culture was incubated. The bright rings are zones of stimulation at low concentration. The particular substance added and other data pertaining to each figure are listed in table 1. The color gives a fairly close approximation to the quality of bacterial luminescence. The difference in brightness of normal and stimulated areas of some cultures made it impossible to allow a correct exposure time for both. In figure 13 the normal area is approximately correct, while the stimulated zone is so over-exposed as to appear white. In figure 14 the exposure was correct for the stimulated zone, and the rest of the plate was thereby so under-exposed that it hardly shows, or appears very blue. The actual exposure-times required for these pictures were many times longer than would be expected if the reciprocity law held throughout such low intensities. In terms of familiar experience, the actual brightness was generally greater than that of a luminous clock dial.

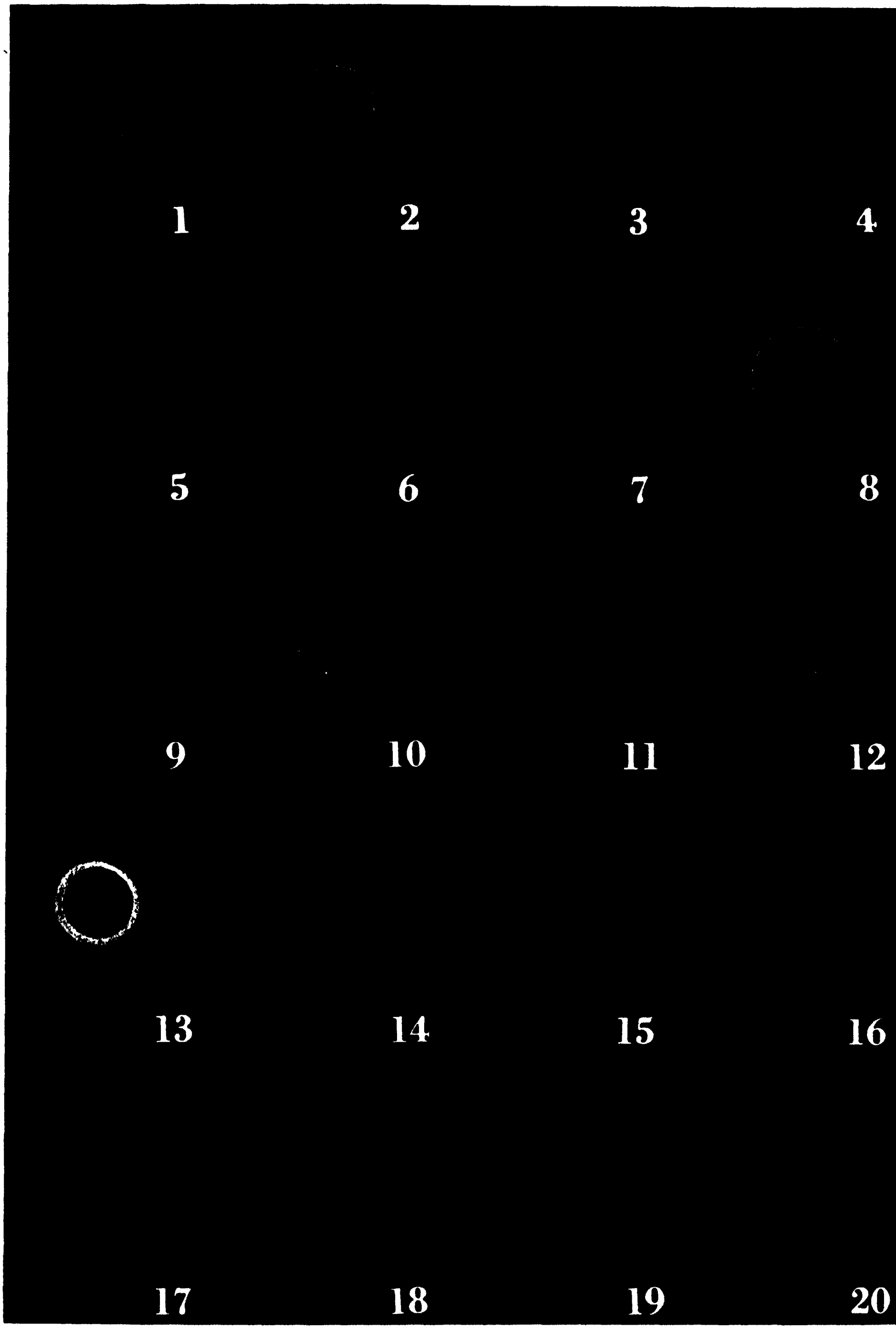

PLATE I

NOTES

METHIONINE MADE AN ESSENTIAL GROWTH FACTOR BY CULTIVATION OF E. COLI IN THE PRESENCE OF METHIONINE AND SULFANILAMIDE

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The strain of *Escherichia coli* chosen for our work was No. 6522, American Type Culture Collection, because it grows well in mediun SG, composed of inorganic salts and glucose (Kohn and Harris, 1941). This characteristic has remained stable for several years, and was not lost when a subculture was transferred daily for several months in medium SG containing (*a*) 1 per cent proteose-peptone, (*b*) amino-acid purine mixture (see below), or (*c*) sulfanilamide gradually increased to 200 mg per cent during the course of a year. When, however, cultivation occurred in the presence of both (*b*) and (*c*), methionine became an essential growth factor, as shown in the following protocol, designed to demonstrate this fact.

EXPERIMENT

The bacteria were grown in medium SG made up to contain *l*-methionine and xanthine at 1×1010^{-5} M, and glycine and *dl*-serine at 4×10^{-5} M, with and without sulfanilamide. The initial sulfanilamide concentration was 2×10^{-3} M, which was gradully increased to 2×10^{-2} M during the course of thirty transfers. The transfers averaged 0.001 ml into 5 ml of medium. Without sulfanilamide the bacteria remained stable throughout, but by the tenth transfer those in sulfanilamide no longer grew in medium SG. The latter, tested in the various amino acid-purine combinations following the thirtieth transfer, were found to grow only in the presence of methionine. Since then the strain has been transferred 20 times in medium SG containing 2×10^{-5} M *dl*-methionine (but no sulfanilamide), and the methionine requirement still remains absolute.

DISCUSSION

Methionine in *E. coli* is a specific antagonist to the sulfonamides (Bliss and Long, 1941; Harris and Kohn, 1941), and the action of other secondary antagonists such as xanthine, serine, and glycine, is dependent upon its presence (Kohn and Harris, 1942). For a variety of reasons, we have argued that the sulfonamides inhibit anabolic reactions, and that of these, the synthesis of methionine is perhaps the first to be affected. The present experiment is consistent with and supports this general line of reasoning.

[1] We wish to thank the Rockefeller Foundation for a grant in aid of this work.

[2] Now on active duty, M.C., U. S. A.

When it is recalled that cultivation in sulfanilamide alone (or in methionine alone) does not change the methionine requirement, the present finding may appear somewhat puzzling. In a general way, the explanation is as follows. Resistance to sulfanilamide developed in a methionine-free medium must involve *inter alia* metabolic adjustments to protect methionine synthesis. Such adjustments need not be made in methionine-containing media.

REFERENCES

Bliss, E. A., and Long, P. H. 1941 The antibacteriostatic action of methionine. Bull. Johns Hopkins Hosp., **69**, 14–38.

Harris, J. S., and Kohn, H. I. 1941 The specific antagonism between methionine and the sulfonamides in Escherichia coli. J. Pharmacol., **73**, 383–400.

Kohn, H. I., and Harris, J. S. 1941 On the mode of action of the sulfonamides. Action on Escherichia coli. J. Pharmacol., **73**, 343–361.

Kohn, H. I., and Harris, J. S. 1942 Purines, amino acids, peptones and pancreas as antagonists and potentiators of sulfonamide in E. coli. J. Pharmacol., in press.

A PRACTICAL SUGGESTION FOR THE SEROLOGICAL TYPE DETERMINATION OF *SALMONELLA* ORGANISMS

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The serological type differentiation of *Salmonella* organisms based on the work by White and Kauffmann gives fast and consistent results. It has become most valuable for epidemiological investigations and lately also as a basis for therapeutic measures. Its practical application, however, has been hampered by the necessity of having large numbers of immune sera, absorbed testing fluids, and type cultures at hand for such work.

Experience with five hundred *Salmonella* infections shows that seventeen serological types of *Salmonella* are of major importance for human pathology in this country. They are enumerated in the following table:

		O-ANTIGEN	H-ANTIGEN Phase 1	H-ANTIGEN Phase 2
Group A.	*S. paratyphi A*	(I), II	a	—
Group B.	*S. paratyphi B*	(I), IV, (V)	b	1, 2
	S. typhi-murium		i	1, 2
	S. chester (-san diego)		e, h	e, n, (x)
	S. derby		f, g	—
Group C_1.	*S. paratyphi C*	VI, VII (Vi)	c	1, 5
	S. choleraesuis	VI, VII	(c)	1, 5
	S. thompson		(k)	1, 5
	S. oranienburg		m, t	—
	S. bareilly		y	1, 5
	S. montevideo		g, m, s	—
Group C_2.	*S. newport*	VI, VIII	e, h	1, 2
	S. muenchen		d	1, 2
Group D.	*Eberthella typhosa*	IX (Vi)	d	—
	S. enteritidis	(I), IX	g, m	—
	S. panama		l, v	1, 5
Group E.	*S. give*	III, X, XXVI	l, v	1, 7
	S. anatum		e, h	1, 6

A satisfactory routine determination of these types can be carried out by slide agglutination with a set of six O-, eleven monophasic H- and a Vi-serum, sufficiently specific for the following antigens or antigen-complexes:

II; IV, V; VI, VII; VI, VIII; IX; III, X, XXVI;
a; b; i; e, h; f, g; m, t; y; g, m, s; d; l, v; 1, 2; Vi

Smooth and well motile cultures are tested first with the O-sera and then with the H-sera of the group. If one of the H-phases is absent, single colonies are

examined. Presence of the Vi-antigen accounts for absence of O-agglutination, as does roughness of the cultures. Monophasic *S. choleraesuis* and thompson strains may be differentiated on the basis of inositol fermentation (thompson ferments inositol).

About 97 per cent of the *Salmonella* cultures from human infections can thus be typed. Most of the other types are agglutinated by some of the sera and can be tentatively identified as *Salmonella*.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK BRANCH

SEMI-ANNUAL MEETING, AGRICULTURAL EXPERIMENT STATION, GENEVA, NEW YORK, OCTOBER 31, 1942

FERMENTATION OF CITRIC ACID BY HOMOFERMENTATIVE LACTIC ACID BACTERIA. *J. J. R. Campbell and I. C. Gunsalus*, Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca.

Certain of the homofermentative lactic acid bacteria, which are known to produce almost exclusively lactic acid from glucose, have been found to utilize citric acid as an energy source. In a tryptone yeast-extract medium, citrate supports growth and is fermented with the production of gas. In contrast to the leuconostocs, these organisms do not require the presence of carbohydrates to attack citrate in growing cultures.

Among the organisms capable of attacking citrate under these conditions are strains of *Streptococcus fecalis*, *Streptococcus liquefaciens*, *Streptococcus zymogenes*, *Lactobacillus delbrueckii*, *Lactobacillus lactis*, and *Lactobacillus casei*.

Fermentation balances on a number of enterococci indicate that under alkaline conditions the products, per mole of citric acid, approach 2 moles of acetic acid, 1 mole of carbon dioxide, and 1 mole of formic acid. Traces of lactic acid are usually present. At more acid reactions neutral volatile products, mainly acetyl—methyl—carbinol, are formed with a corresponding decrease in the yield of volatile acids.

NITRATE IN PLANTS AND ITS SIGNIFICANCE IN FERMENTATION. *J. K. Wilson*, College of Agriculture, Cornell University, Ithaca.

DIFFICULTIES IN DEFINING THE GENUS ALCALIGENES. *H. J. Conn*, N. Y. State Agricultural Experiment Station, Geneva.

The present study was undertaken to learn whether certain soil organisms belong in the genus *Alcaligenes*. When the type species *Alcaligenes faecalis* Castellani and Chambers (*Bacillus faecalis alcaligenes* Petruschky) was investigated, however, it was found that the type species is not well characterized and, therefore, that the genus is ill-defined. Petruschky originally described his organism as a peritrichous rod from feces that yielded neither acid nor gas from carbohydrate media, but produced alkalinity in milk. This study shows that at least three species are being distributed as *Alcaligenes faecalis:* 1) a peritrichous rod requiring organic nitrogen, 2) a lophotrichous rod requiring organic nitrogen, and 3) a peritrichous rod using nitrate or ammonium salts as sole sources of nitrogen. It seems probable that No. 1 is the organism originally described by Petruschky. This organism is very similar to *Bacterium bronchisepticum*. No. 2 is excluded because it has lophotrichous flagella. No. 3 agrees with Petruschky's description and has been distributed by type-culture collections, but as its fecal origin is doubtful, and it grows on synthetic media, it probably belongs to a different genus. It is concluded that strains recovered from soil that do not produce acid or gas from carbohydrate media are related to No. 3, not to No. 1.

THE DEMONSTRATION OF ACID-FAST ORGANISMS BY FLUORESCENCE MICROSCOPY. *Oscar W. Richards*, Spencer Lens Company, Buffalo.

Carbol-auramin and carbol-thioflavine are selectively retained by acid-fast organisms in slide preparations decolorized by acid alcohol. The organisms fluoresce on exposure to ultraviolet radiation, appearing yellow. The strong contrast between the organisms and the dark background makes possible the recognition of acid-fast organisms at lower magnifications than other staining methods, and therefore larger visual fields can be utilized. *Mycobacterium tuberculosis* is readily seen in smear and tissue section preparations and the fluo-

rescence technic is more sensitive than the Ziehl-Neelsen method. *Actinomyces scabies* has been shown between the cells of the diseased potato tubers. *Mycobacterium leprae*, *Mycobacterium paratuberculosis* (Bacillus of Johne's disease), and a number of non-pathogenic, acid-fast bacteria are also readily demonstrated. Tissue sections should be mounted in glycerole, for balsam, clarite, and damar are fluorescent and cause rapid fading of the dye. Semi-permanent preparations are made by ringing the cover glass with gold size or asphaltum. Faded preparations can be restained to their original brilliance. The bacteria and fungous filaments are easily found in the tissue sections and the autofluorescence of the tissue proteins is often sufficient for histological localization.

FLUORESCENCE MICROSCOPY IN BACTERIOLOGY. *E. Loewenstein and G. Crossman*, Bausch and Lomb Optical Company, Rochester.

ON THE OCCURRENCE OF TYPE VI PNEUMOCOCCAL ANTIBODIES IN ANTI-HEMOPHILUS INFLUENZAE HORSE SERUM. *Erwin Neter*, University of Buffalo and Children's Hospital, Buffalo.

When it was found that spinal fluid from a patient with Type VI pneumococcal meningitis contained an antigen which reacted with anti-*Hemophilus influenzae* horse serum, this investigation was undertaken to learn whether pneumococcal antibodies were present in other samples of such sera. Accordingly, samples representing ten different anti-*Hemophilus influenzae* horse sera were obtained from Dr. Leah R. Seidman, Boston, and tested for the presence of precipitins and agglutinins employing the strain of pneumococcus isolated from the above patient. Seven of the sera reacted strongly, two gave weak or irregular results, and one was negative. Zone phenomena were not encountered. Controls employing normal and antipneumococcal horse sera were negative. Moreover, ten other strains of Type VI pneumococcus, obtained through the courtesy of Dr. A. W. Walter, New York, and Dr. Harold W. Lyall, Albany, likewise reacted with anti-*Hemophilus influenzae* horse sera. On the other hand, no agglutination occurred when three strains of Type B *Hemophilus influenzae* were tested with Type VI antipneumococcal serum and when Type VI pneumococci were tested with anti-Type B *Hemophilus influenzae* rabbit sera. It remains to be determined whether the antibodies against Type VI pneumococcus in the anti-*Hemophilus influenzae* horse sera originate from antigenic components of *Hemophilus influenzae*, from the medium used for cultivation, or from other sources.

PORCINE BRUCELLOSIS IN NEW YORK STATE. *W. S. Stone*, Veterinary College, Cornell University, Ithaca.

UNEXPECTED ACTION OF SOME DISINFECTANTS. *Otto Rahn*, College of Agriculture, Cornell University, Ithaca.

PROPOSED CHANGES IN THE OUTLINE CLASSIFICATION FOR THE NEXT EDITION OF THE BERGEY MANUAL. *Robert S. Breed*, N. Y. State Agricultural Experiment Station, Geneva.

BACTERIAL DEATH RATES AND DECIMAL REDUCTION TIME. *Leonard I. Katzin and L. A. Sandholzer*, U. S. Public Health Service, National Institute of Health.

A NEW COCCIDIUM, EIMERIA BRUNETTI, N.SP., PATHOGENIC FOR CHICKENS. *P. P. Levine*, New York State Veterinary College, Cornell University, Ithaca.

Eimeria brunetti, a new species of coccidium pathogenic for the chicken, is herewith described. Sporulated oöcysts range in size from 18.1–24.2 μ in width x 20.7–30.3 μ, the average being 21.7 x 26.8 μ. Oöcysts appear in the feces at the end of the fifth day after the infective feeding. Oöcysts and developmental forms are present in the lower half of the small intestine and in the rectum, cloaca and caeca. In severe infections, the upper half of the small intestine may also be involved. The oöcysts are discharged periodically, the greatest elimination being between 9 a.m. and 3 p.m. No gross lesions are found in light infections. Moderately heavy infections produce a catarrhal enteritis with blood-tinged mucous exudate. Quite often there

appear on the mucosa short, red, horizontal streaks that extend as ladder-like rows along the length of the affected parts. Coagulation necrosis and sloughing of the mucosa occur in severe infections. Frequently the necrotic mucosa takes the form of a dry, diphtheritic membrane lining the intestinal tract. These lesions may be confluent or focal. The dilated portion of the caeca is usually without lesions aside from erythema of the mucosal ridges. On the other hand, the constricted, proximal, tubular entrances to the caecal pouches become markedly dilated and plugged by short caseous cores. Cross-immunity tests indicate that *Eimeria brunetti* is different from previously described species with which it might be confused. Sulfaguanidine, when fed to chickens in the proportion of 0.5 per cent of the ration, prevented infection. A natural outbreak in a commercial flock of coccidiosis, proven to have been caused in part by *Eimeria brunetti*, has been studied.

THE USE OF THE ELECTRON MICROSCOPE IN THE STUDY OF THE STRUCTURE OF THE BACTERIAL CELL. *Georges Knaysi*, College of Agriculture, Cornell University, Ithaca.

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